Sulfur and the Origins of Life

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Jonathan Hill

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Abstract

Both organic and inorganic sulfur play an important role in fundamental contemporary biochemistry, suggesting that life's common ancestor used sulfur in its metabolism. In support of this idea is the widely held belief that present-day sulfur-metabolising thermophilic bacteria are the most primitive organisms within the biosphere. This thesis postulates that sulfur may have been important in the origins of life, and reports investigates several interesting and potentially prebiotic reactions involving sulfur.

Three different types of chemistry were investigated. The formation of pyrophosphate from phosphate with thioesters over metal phosphate precipitates has been reported; this can be considered a biomimic of the important succinyl-CoA synthetase-catalysed reaction in the citric acid cycle of present-day organisms. The ability of ferrous sulfide and/or ferrous phosphate precipitates to catalyse pyrophosphate production with thioesters or metal ion-bound thioacids as condensing agents was investigated. No pyrophosphate production was detected, but interesting results regarding the effect of ferrous ions in solution and ferrous sulfide precipitates and ferrous phosphate precipitates on the rate of hydrolysis of thioacetic acid are reported.

Ferrous ion- and sulfide ion-mediated redox chemistry of thioesters and metal-ion bound thioacids was investigated. The motivation for these experiments came from the involvement of thioesters in contemporary biochemical redox reactions. No ferrous ion- or sulfide ion-dependent redox chemistry was demonstrated.

Finally amide bond formation between amines and metal ion-bound thioacids was investigated. It was discovered that nickel sulfide precipitates are efficient catalysts in this process. Nickel sulfide would have been present in the oceans of primordial earth, and may therefore have been an important prebiotic catalyst in the origin of amide bonds in metabolism.

ABBREVIATIONS

ADP	adenosine 5'-diphosphate
ATP	adenosine 5'triphosphate
bs	broad singlet
2,4-DNP	2,4-dinitrophenyl hydrazine
CoA	coenzyme A
d	doublet
GDP	guanosine 5'-diphosphate
GTP	guanosine 5'-triphosphate
HEPES	4-(*N*-2-hydroxyethyl)-1-piperazine-*N*-2'-ethane sulphonic acid
HPLC	High pressure liquid chromatography
hrs	hours
M	moles per litre
MES	4-(*N*-morpholine)ethane-sulphonic acid
mg	milligrams
min	minutes
mL	millilitres
mM	millimoles per litre
mmol	millimoles
mol	moles
MS	mass spectroscopy
o	octet
P_i	inorganic phosphate
PIPES	1,4-piperazine-*N,N*-bis(2-ethane sulfonic acid)
PP_i	inorganic pyrophosphate
q	quartet
rpm	revolutions per minute
s	singlet
t	triplet

TLC	thin layer chromatography
v/v	volume/volume
Z	carbobenzyloxy

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Chapter 1 Introduction

1.1 The nature of origins of life research

This thesis investigates the possible role of sulfur in the origins of life. It is not possible to reconstruct the exact series of events that led to the origin of life as we know it, but by drawing upon biochemical and geochemical data we can formulate testable hypotheses as to what type of chemistry may have been involved in life's creation. Before continuing with a discussion of the nature of origins of life research, two important postulates, made by most workers in the field, must be outlined.

The first postulate is that terrestrial life arose on earth, or in an environment similar to one of those present on prebiotic earth. (The term "origin of life" may henceforth be taken to imply "origin of life on earth".) This assumption is useful because it allows the prediction of the various physical conditions in which life may have arisen. This in turn places limitations on the types of chemistry that may have contributed to the origins of life, thereby allowing the more rigorous testing of theories. The idea that life may have originated elsewhere in the universe and come to earth from outer space has its proponents and deserves mention here, but will not be considered further in this discussion as it does not address the problem of the ultimate origin of life.

A second postulate that is often made is that all modern-day life on earth has arisen from a common ancestor. From this it follows that similarities in the fundamental biochemistry of all organisms (for example, the involvement of acetyl-CoA, ATP and the TCA cycle in living systems) reflects divergent evolution from some ancestral life form, as opposed to convergent evolution from different lineages. This allows us to extrapolate back from contemporary metabolism, particularly the more ubiquitous and fundamental biochemical processes, and predict the types of chemistry that may have been used by life's common ancestor. This idea has been called "congruence" by De Duve[1], and allows a Popperian assessment of theories based on their "explanatory power", as highlighted by Wächtershäuser[2].

Taken together, the above two postulates state that the ubiquitous types of chemistry seen in all living systems today was present in life's common ancestor, and that this common ancestor arose on earth itself. It follows from this that fundamental metabolism, or one involving similar types of chemistry, arose on prebiotic earth. If it is assumed that metabolic systems have evolved in a Darwinian fashion, it should be possible to see remnants of ancestral metabolic chemistry in the ubiquitous and fundamental types of biochemistry occurring in the present-day biosphere.

These biochemical and geochemical clues allow researchers to perform abiotic experiments that mimic fundamental biochemical processes (termed "biomimetic" systems) using potentially prebiotic reagents under primordial reaction conditions. In this way, this type of research addresses the question of how life arose on earth, as well as generating information regarding fundamental aspects of some of the chemistry that is occurring in living systems today.

1.2 Setting the stage

Plausibly prebiotic experiments must be limited to a set of possible conditions present on earth during the origin of life. The physical conditions on earth have changed drastically since its formation[3] around 4.5 billion years ago (Ga), and it is therefore necessary to determine over what time period life probably arose, in order to make predictions regarding the environmental conditions prevailing on earth at this time.

Stromatolites that resemble the morphology of present-day cyanobacteria have been found in 3.5 billion-year-old rocks in Australia. An enrichment of the ^{12}C isotope over the heavier ^{13}C isotope was detected in organic compounds isolated from these fossils, which is taken as an indicator of catalytic carbon fixation, presumably through biological activity[4]. Possible evidence for the earlier existence of life on earth comes from microfossils found in 3.8 billion-year-old rocks from Greenland[5, 6], although the evidence is not as definitive as the stromatolite findings. It can be concluded, then, that life was present on earth within one billion years of its formation, and possibly within 700 million years.

Life is an aqueous phenomenon, and it can be assumed that water was involved as a solvent during the origins of life. Oceans would have formed as the young earth cooled[7], but frequent meteoritic impacts capable of vaporising oceans and sterilising the planet are believed to have occurred up until around 4 Ga[8]; this therefore represents an upper limit for the time of life's origin.

The origin of life on earth is therefore considered to have occurred between approximately 3.5 and 4 Ga. At this time the oceans would have been slightly acidic due to dissolved carbon dioxide, hydrogen chloride and sulfur dioxide, and would have contained reducing mixtures of sulfides and possibly hydrogen. The average temperature of the oceans is uncertain[9], but there would have been local hotspots associated with hydrothermal activity as well as cooler regions[10].

The atmospheric composition of prebiotic earth is a contentious issue. The dominant view in recent years has been that the primitive atmosphere was a weakly reducing mixture of CO_2, N_2 and H_2O, combined with lesser amounts of CO and H_2[11]. The high concentration of oxygen in today's atmosphere is as a result of biological activity, and so would not have been present on prebiotic earth. (Furthermore, any traces of oxygen would have reacted with metals or hydrogen during earths formation.) The ozone layer would not have been present at this time either, and large amounts of UV light would therefore have reached the earth's surface and penetrated oceanic waters to a depth of several metres. UV light causes the degradation of organic compounds, and so its presence at high intensities on the surface of prebiotic earth make these exposed surface environments less likely as possible sites for the origin of life.

One likely setting for the origin of life is environments associated with deep-sea hydrothermal vent systems[12]. These occur at fissures in the earths crust on the ocean floor, where sea-water percolates down through the crust and is ejected as a heated sulfide-laden solution. They are therefore characterised by large amounts of metal sulfide precipitates[13], particularly iron and to a lesser extent nickel, tungsten and molybdenum, and generate a temperature gradient in the surrounding water.

Contemporary hydrothermal vent systems offer a type of environment that, in principle, has not changed during the four billion years that have passed since the earths crust was formed[10]. Several sulfur-metabolising thermophilic bacterial species have been recently isolated from deep-sea hydrothermal vents, and phylogenetic analyses suggest that these species are some of the most archaic in the biosphere[14]. This result lends support to the idea that life may have found its beginnings in such a setting.

1.3 S and the origins of life

This thesis focuses on the possible involvement of sulfur in the origins of life, both as inorganic metal sulfide deposits and as a component of organic molecules. The following sections outline the involvement of organic and inorganic sulfur in contemporary biochemistry, and then introduce some potentially prebiotic biomimetic reactions involving sulfur.

1.3.1 Organic sulfur in metabolism

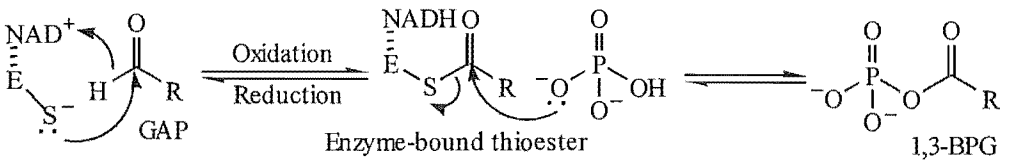
Organic sulfur serves many functions in contemporary biochemistry. One of the most significant is the activation of carboxylic acids as thioesters. Thioesters can be thought of as activated carboxylic acids, with the textbook example being acetyl coenzyme A (see figure 2.6). A ubiquitous thioester involved in the fundamental biochemistry of all life as we know it, acetyl-CoA plays a central role in both anabolic and catabolic metabolism.

The pantetheine functionality, comprising part of the acetyl-CoA molecule, is an important thioester-forming moiety in biochemistry, and forms the functional part of many other important biological molecules. Acyl carrier protein (ACP) is one such example; it provides activated building blocks in the analogous processes of fatty acid biosynthesis and non-ribosomal peptide biosynthesis. The involvement of thioesters in non-ribosomal peptide biosynthesis is discussed further in chapter 4.

Thioester bonds and phosphoanhydride linkages are both high-energy bonds that are freely converted in metabolism. This potential for interconversion arises because the

amount of energy stored within the two bond types is very similar: the free energy for hydrolysis of thioesters and phosphoanhydrides is -41.5kJmol^{-1} and –40.5kJmol^{-1}, respectively. Adenosine triphosphate (ATP) is a biochemically ubiquitous phosphoanhydride that plays a central role in cellular energy transport and utilisation, sometimes being called the "cellular energy currency". The fact that thioesters can directly interconvert with ATP (or an equivalent species) makes them very useful reagents in biochemistry.

Thioesters also participate in biological redox reactions, and therefore can be considered as key intermediates in the interconversion between electron-linked and group-linked energy[1]. An example of thioesters fulfilling this role can be seen in the reaction catalysed by glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In glycolysis, GAPDH catalyses the oxidation of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate (figure 1.1), and catalyses the reverse reaction in the reductive photosynthetic and gluconeogenic pathways. A thioester species is involved as an intermediate, and the reaction mechanism echoes the theme of the last paragraph, namely, the interconversion of phosphoanhydride and thioester bonds.



E = Enzyme
R = C(OH)CH $_2$CH$_2$OPO$_3^{2-}$
GAP = Glyceraldehyde-3-phosphate
1,3-BPG = 1,3 Bisphospho glycerate

Figure 1.1: A simplified reaction mechanism of glyceraldehyde-3-phosphate

The redox chemistry of thioesters is also coupled to the synthesis and cleavage of carbon-carbon bonds within metabolism. An illustrative example of this type of chemistry is the reaction catalysed by the pyruvate dehydrogenase multienzyme complex (PDH) (figure 1.2). This represents the last step in glycolysis, and involves

the oxidative decarboxylation of pyruvate to generate activated acetate in the form of acetyl-CoA, which is then used in the TCA cycle.

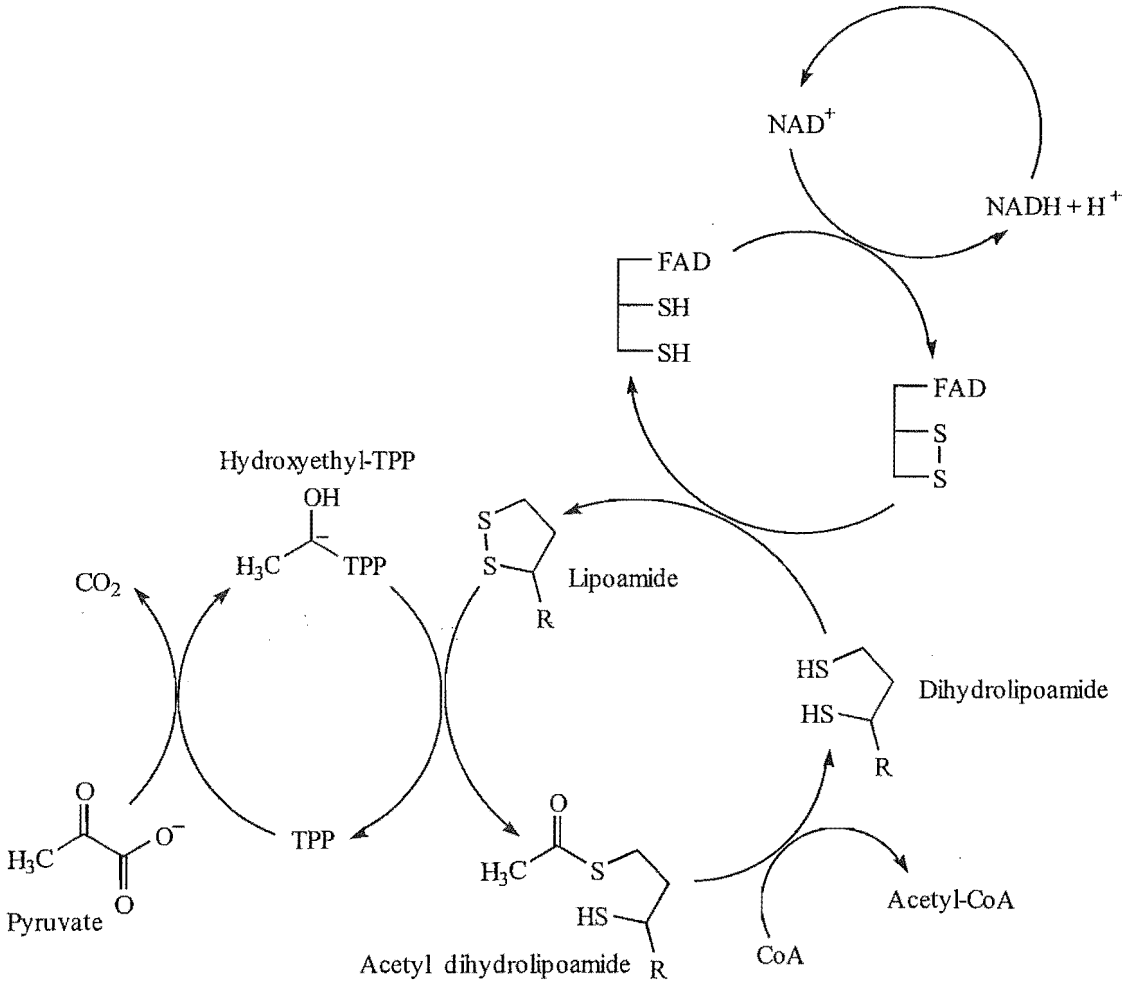


Figure 1.2: The pyruvate dehydrogenase multienzyme complex reaction mechanism

Biological reductive carbon fixation using thioesters (essentially the reverse type of the PDH-catalysed chemistry) is also seen. In the reductive citric acid cycle, used by certain bacterial species to fix carbon, thioesters are used as substrates in two of the four reductive carboxylation steps.

The PDH catalysed reactions (figure 1.2) involve the flow of electrons through sulfide containing molecules to give disulfide intermediates. Sulfide/disulfide redox couples are used extensively in metabolism, and represent another important use of sulfur in

biochemistry. A ubiquitous biomolecule of central importance in redox processes within cells and utilising sulfide/disulfide chemistry is glutathione (figure 1.3).

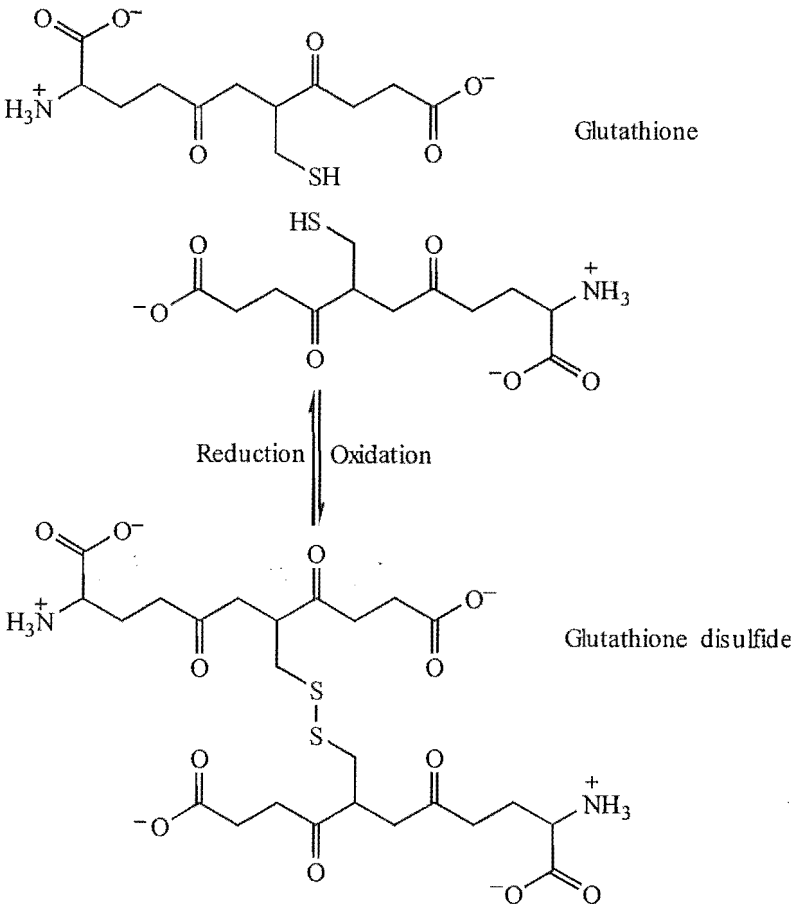


Figure 1.3: The redox chemistry of glutathione

Finally, sulfide ions and thiols are also used as nucleophiles in catalytic mechanisms; cysteine residues are present in the active sites of many enzymes.

1.3.2 Inorganic sulfur in metabolism

One of the most fundamental roles of sulfur in present-day metabolism is as a structural component within iron-sulfur clusters, believed by some to be the first electron-transfer groups to be produced during chemical evolution[15]. These clusters, bound to proteins through coordinated cysteine residues (figure 1.4) are ultimate donors or acceptors of electrons involved in cellular redox processes.

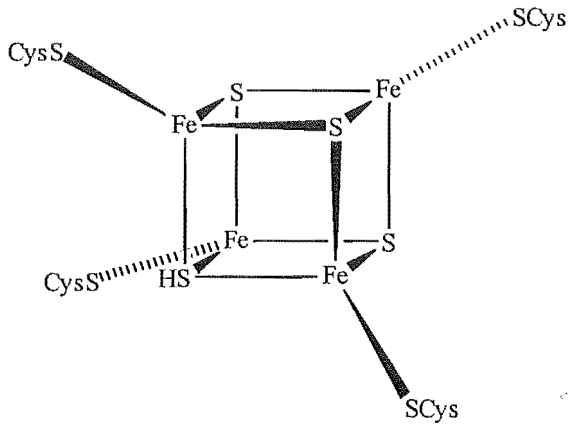


Figure 1.4: Basic structure of the protein-bound Fe_4S_4 cluster

The iron centres are the redox-active component of these clusters, with sulfur atoms providing structural integrity, both by holding the metal ions in close proximity to each other and allowing proteins to bond with and "embrace" the clusters. In this way, sulfur can be thought of as bridging biological organic and inorganic chemistries.

1.3.3 Sulfur in Geochemistry

Reduced sulfur is present in high concentrations in the earth's core. At rifts in the earths crust hydrogen sulfide is expelled, and leads to the precipitation of metal sulfides, particularly black iron sulfide, around deep-sea hydrothermal vents. These vent systems have already been discussed as potential sites for the origin of life. The metal sulfide precipitates are in principle little different to biological iron-sulfur clusters, and provide a surface that can bind charged molecules. This binding may have led to catalysis of reactions involved in the origin of life. The effect of metal sulfide precipitates on several potentially prebiotic reactions is discussed in subsequent sections and chapters where relevant.

1.3.4 S species implicated in origin's of life theories

Christian de Duve, a respected biologist and Nobel laureate, has proposed a pivotal role for thioesters in the origin of life[1]. In his "thioester world" hypothesis the spontaneous ligation of thioesters, a phenomenon first discovered by Wieland in 1988[16], gives rise to a rich and varied combination of catalytically-active polypeptide-

like "multimers". It is possible that autocatalytic cycles and self-organisation could have arisen spontaneously from such catalytic complexity[17]. It has been postulated by de Duve that thioesters could have been formed prebiotically by the oxidative condensation of thiols with aldehydes or α-keto acids; an idea that receives experimental support from a paper reported by Weber[18], which will be discussed further in chapter 3.

In contrast to de Duve, Michael Russell has approached the problem of the origin of life from a geological background, and sees iron sulfide precipitates as playing a central role in this process[13, 19, 20]. He has postulated that life emerged from growing aggregates of iron-sulfur bubbles, or "probotryoids", which could have formed when alkaline and highly reducing hydrothermal solutions met with the mildly oxidising, acidic and iron-bearing Hadean ocean water in deep-sea hydrothermal systems. Laboratory experiments and geological findings have shown that these probotryoids were likely to have been formed on prebiotic earth. He has hypothesised that the cationic surfaces of the iron sulfur precipitates could have bound anionic organic molecules and catalysed prebiotic reactions. He also acknowledges the possible involvement of oxidative pyrite (FeS_2) formation from iron sulfide (FeS) as a source of reducing power[21], which is a phenomenon that plays a central role in the origin of life theories of Gunter Wächtershäuser.

Wächtershäuser has proposed an autotrophic origin of life theory, with the oxidative formation of pyrite from iron sulfide and hydrogen sulfide (demonstrated under anaerobic and potentially prebiotic conditions[22]) providing the driving force for reductive carbon fixation[23]. He has theorised a pyrite-pulled carbon-fixing autocatalytic cycle that resembles the reductive TCA cycle, seen in some present-day bacteria, as the first metabolic system[24], and demonstrated several biomimetic redox reactions using the FeS/H_2S reductant[25-27]. Organic substrates and intermediates are thought to bind to the metal sulfide precipitates as thiols and thioacids. Wachtershauser's suggestion that early life forms may have utilised a pyrite-forming metabolism is supported by the recent discovery of pyritic filamentous residues, believed to be microfossils, in a 3.25 Ga Australian deep-sea volcanogenic massive sulfide deposit[28].

1.3.5 Biomimetic Chemistry

The postulates highlighted at the start of this chapter, that all life on earth today has evolved from a common ancestor that itself arose on prebiotic earth, allow us to assess the plausibility of origin of life theories by extrapolating back from contemporary metabolism. By the principle of congruence, origin of life theories should be able to explain features of fundamental biochemistry. This is the argument that de Duve use's in favour of his "thioester world" hypothesis[1], which draws support from the extensive involvement of thioesters in present-day metabolism. In particular, his mechanism for the formation of "multimers" is analogous to non-ribosomal peptide biosynthesis.

Russell's iron-sulfur-based theories draw support from the important role played by FeS proteins in contemporary metabolism, and the archaic nature of these proteins. The binding of prebiotic substrates to a cationic catalyst offers an explanation as to why biochemical organic molecules tend to be anionic. Also, probotryoids, the key players in his theory, are membranous aggregates that will bud to form new probotryoids, analogous to cell division in present-day biology.

Iron-sulfur clusters are also key players in Wächterschäuser's origin of life theories. Interestingly, the inorganic FeS/FeS_2 redox couple that plays a central role in his hypotheses can be considered analogous to the biological S^{2-}/S_2^{2-} redox couple. Furthermore, metal ion-bound thioacids have been proposed as intermediates in some potentially prebiotic reactions. Metal-bound thioacids can be thought of as thioester analogues (figure 1.5), and may be the evolutionary precursors of the thioesters seen in modern-day biochemistry.

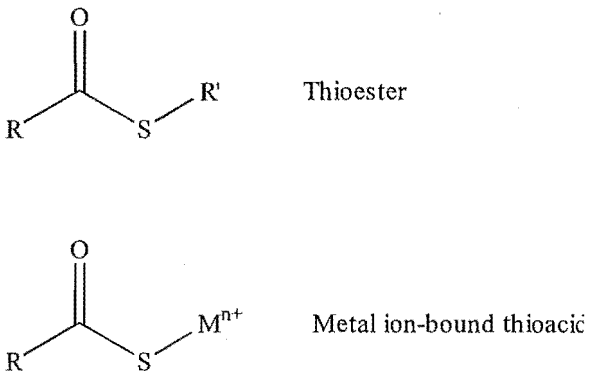


Figure 1.5: A structural comparison of thioesters and metal ion-bound thioacids

1.4 Summary

This thesis reports an investigation into three types of chemistry mediated by iron and sulfur and involving thioesters and metal ion-bound thioesters as substrates. Chapter 2 discusses possible catalysis by ferrous phosphate and/or ferrous sulfide precipitates in the formation of phosphoanhydride bonds with thioesters or as dehydrating agents; chapter 3 discusses the ferrous- and sulfide-mediated redox chemistry of thioesters and thioacids; and the experiments in chapter 4 are involved with iron and nickel-mediated formation of amide bonds between amines and thioacids.

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Chapter 2 Dehydration chemistry

2.1 Introduction

ATP (Figure 2.1) can be equated with generic cellular chemical energy, and is often referred to as the "cellular energy currency". All metabolism is heavily dependent on reactions involving ATP; exergonic reactions are often coupled to the synthesis of high-energy ATP phosphoanhydride bonds, and the cleavage of these bonds is then used to drive endergonic processes (Figure 2.1).

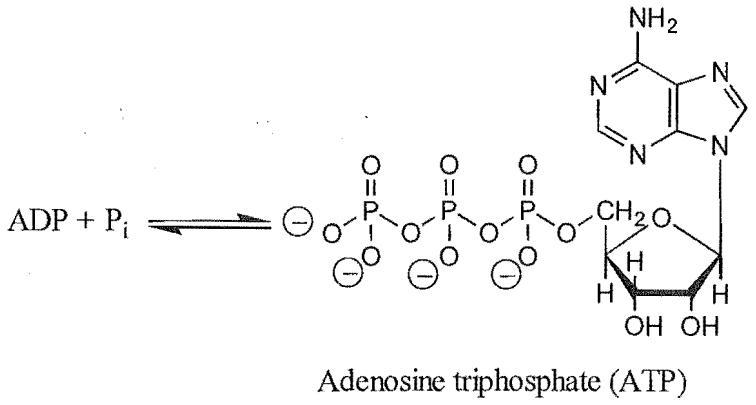


Figure 2.1: The synthesis and cleavage of ATP

The central role played by phosphoanhydride chemistry in contemporary metabolism suggests that polyphosphates may have been involved in the metabolism of life's common ancestor, and may have been involved in the origin of life[1-4]. ATP is a relatively complex biomolecule, so simpler polyphosphate species may have played an important role in the origin of life. Inorganic polyphosphates are ubiquitous biochemicals[1] which play an important role in the metabolism of many present-day organisms, and are more plausible prebiotic reagents than ATP. Pyrophosphate is the simplest example of an inorganic polyphosphate (Figure 2.2), and is generally believed to have preceeded ATP as purveyor of energy in ancestral metabolic systems[2, 3]. Efficient prebiotic production of polyphosphates has been the subject of

much research. Polyphosphate synthesis from orthophosphate monomers requires dehydration chemistry, which is unfavourable in aqueous environments. Several potentially prebiotic syntheses of polyphosphates have been reported[5-11], but none represent a robust mechanism for polyphosphate synthesis on prebiotic earth. . One other potential source of polyphosphates on prebiotic earth is volcanic activity[12], where polyphosphates are believed to be produced by high temperature reactions. There is scepticism, however, as to the involvement of this source of inorganic polyphosphates in the origin of life[13].

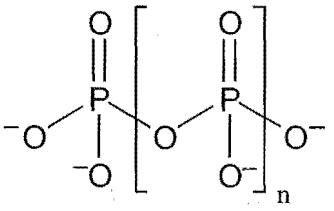


Figure 2.2: The structure of pyrophosphate and other polyphosphates

ATP is today biosynthesised by two mechanisms: substrate-level phosphorylation and oxidative phosphorylation. Oxidative phosphorylation uses the energy released by flow of electrons through a membrane-bound electron transport chain to synthesise ATP; this is a relatively complex biosynthetic pathway. Substrate-level phosphorylation, on the other hand, involves direct reaction of metabolites, such as the use of thioesters as condensing agents. Contemporary metabolism is therefore heavily dependent on the interconversion of thioesters and phosphoanhydrides (as was outlined in section 1.2.1). Of relevance to this chapter is the involvement of thioesters in living systems as condensing agents in the formation of phosphoanhydride bonds. A biological example of this type of chemistry is the reversible reaction catalysed by succinyl-CoA synthetase (Figure 2.3), where a phosphoanhydride bond is formed at the expense of a thioester bond.

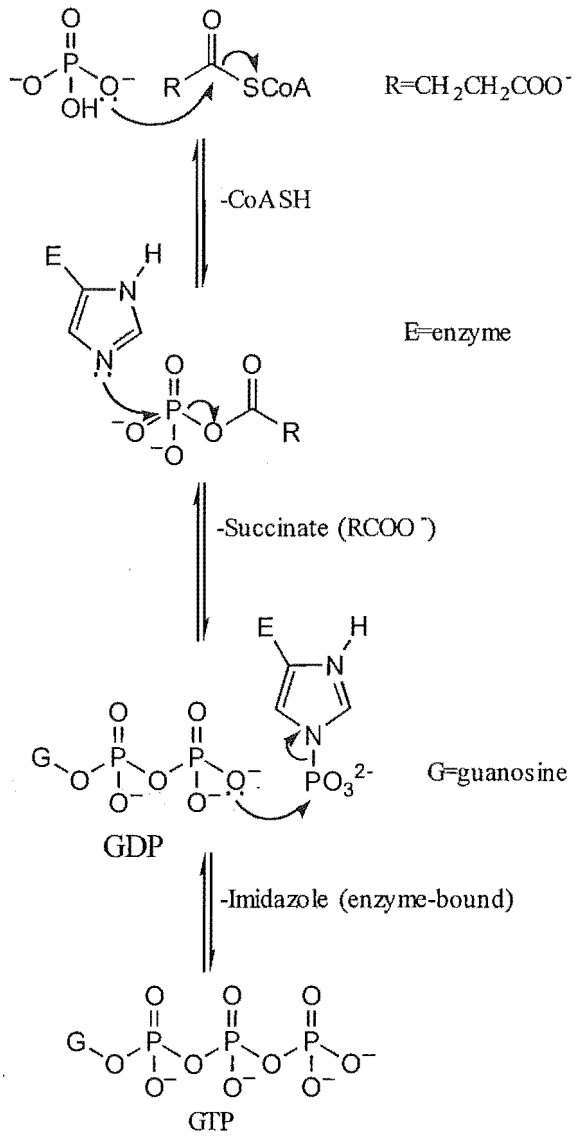


Figure 2.3: The reaction mechanism of succinyl-CoA synthetase

Weber has reported a biomimetic analogue of this process, involving the production of pyrophosphate from inorganic phosphate with *N,S*-diacetyl cysteamine as the condensing agent[6] (figure 2.4). Weber's experiment was carried out at room temperature with hydroxyapatite and imidazole as catalysts; after 6 days virtually all the thioester had reacted and 11% pyrophosphate (based on thioester consumption) had been formed. Hydroxyapatite is the most common geological phosphate mineral ($Ca_5(OH)(PO_4)_3$) and imidazole is a plausible prebiotic agent (interestingly, succinyl-CoA synthetase utilises an imidazole group in catalysis in the form of a catalytic histidine residue). Weber's proposed reaction mechanism for pyrophosphate production is given below (figure 2.4).

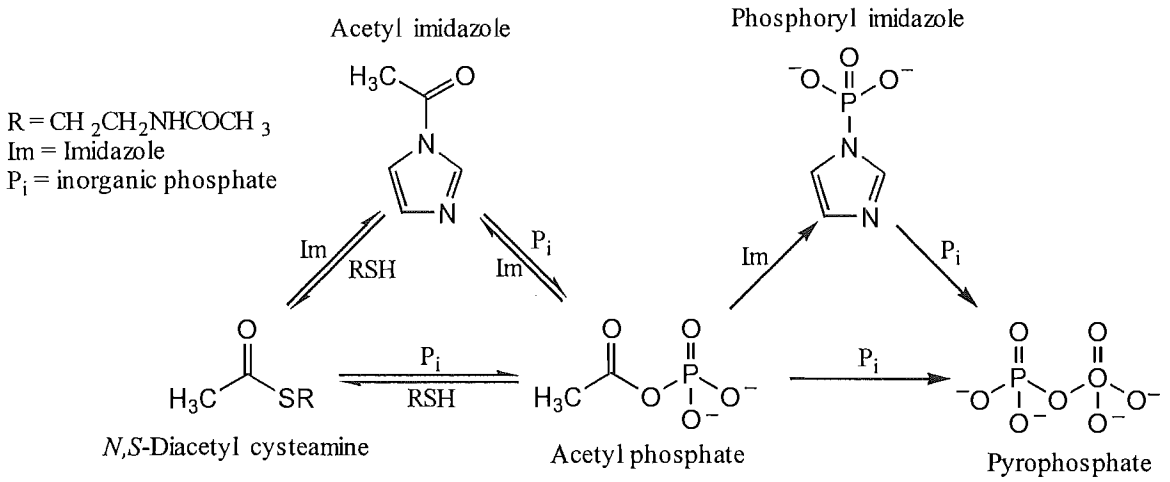


Figure 2.4: Weber's proposed reaction mechanism for pyrophosphate production

Both Weber's reaction and the succinyl-CoA synthetase-catalysed reaction involve acyl phosphates; acyl phosphates are obligatory intermediates in all interconversions between thioesters and phosphoanhydrides.

Catalysis of the second step in this process has been investigated by de Zwart[14]. He has reported that pyrophosphate is formed in up to 25% yield from mixtures containing acetyl phosphate, phosphate and ferrous ions (figure 2.5). Both ferrous phosphate precipitates and ferrous phosphate/ferrous sulfide coprecipitates were shown to be effective catalysts in the reaction (which occurred in the absence of amine nucleophiles). If ferrous sulfide and/or ferrous phosphate could also catalyse the formation of acyl phosphates from thioesters, or thioester-like species, it may be possible that such precipitates might catalyse the overall formation of pyrophosphate from thioesters as condensing agents (figure 2.5) by analogy with the Weber experiment.

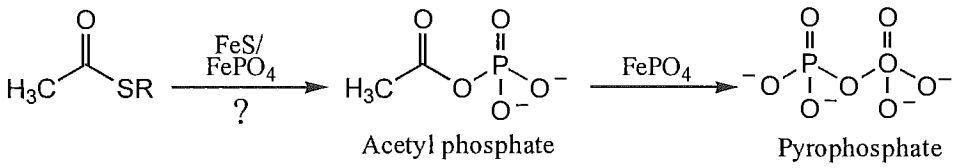


Figure 2.5: The hypothesised production of pyrophosphate from thioesters or thioester-like species with a ferrous phosphate catalyst

The overall aims of the experiments described in this chapter were to evaluate the possibility of pyrophosphate production from phosphate in the presence of $FeS/FePO_4$ coprecipitates with thioester-type species as condensing agents.

2.2 Reaction details

2.2.1 Choice of thioesters and thioacids

N,S-diacetyl cysteamine was chosen as the thioester to be used in these experiments because it mimics the active functional group of acetyl-CoA, a molecule of central importance in contemporary metabolism (see section 1.2.1) (figure 2.6). Thioacetic acid was also investigated as a potential condensing agent bcause, as was discussed in section 1.3.5, metal-bound thioacids can be thought of as thioester mimics (and possibly their evolutionary precursors in metabolism). Thioacetic acid was chosen as the specific thioacid to be used because it is the analogous thioacid analogue to acetyl-CoA (figure 2.6). It was hoped that the thioacetic acid would bind to the FeS precipitate surface and participate in phosphorolysis reactions with coprecipitated $FePO_4$.

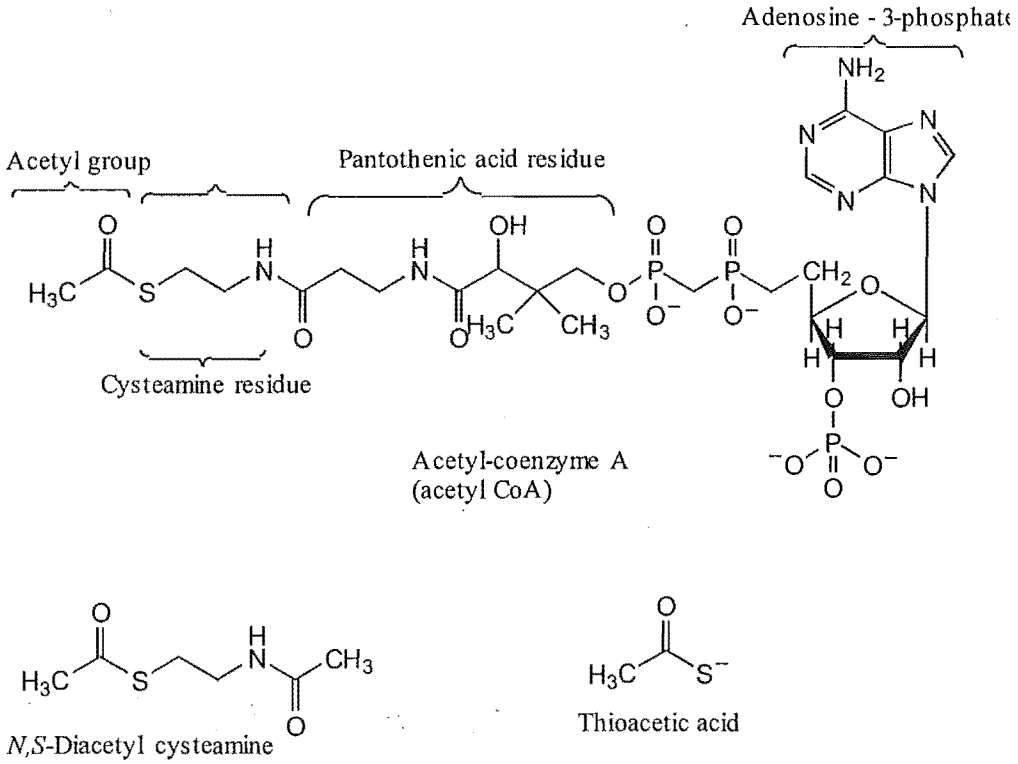


Figure 2.6: The structures of acetyl-CoA, *N,S*-diacetyl cysteamine and thioacetic acid

N,S-Diacetyl cysteamine was synthesised by the acetylation of cysteamine with acetic anhydride (figure 2.7) by the method of Schwab and Klassen[15]. The ester was prepared in 98% yield, and was identical to an authentic sample as determined by ^{1}H NMR analysis.

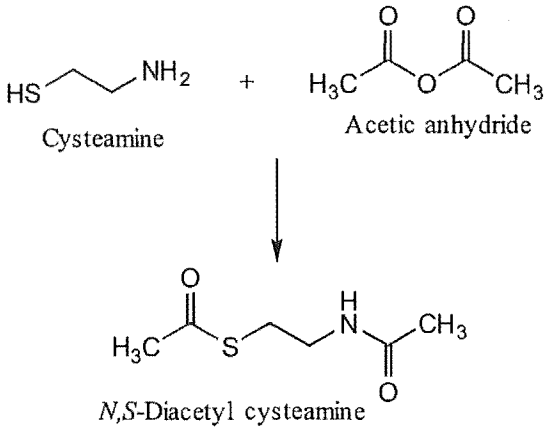


Figure 2.7: Synthesis of *N,S*-diacetyl cysteamine

2.2.2 The reaction system

Solutions of *N,S*-diacetyl cysteamine or thioacetic acid were incubated in aqueous mixtures containing $FePO_4$ and/or FeS precipitates. MES or Bis-TRIS buffers were used to maintain the pH of reaction mixtures at 6.5, which was shown by de Zwart to be an optimum pH for phosphorylitic attack on acetyl phosphate in his experiments.

The relevant reactions that might occur in this reaction system are shown below in figure 2.8.

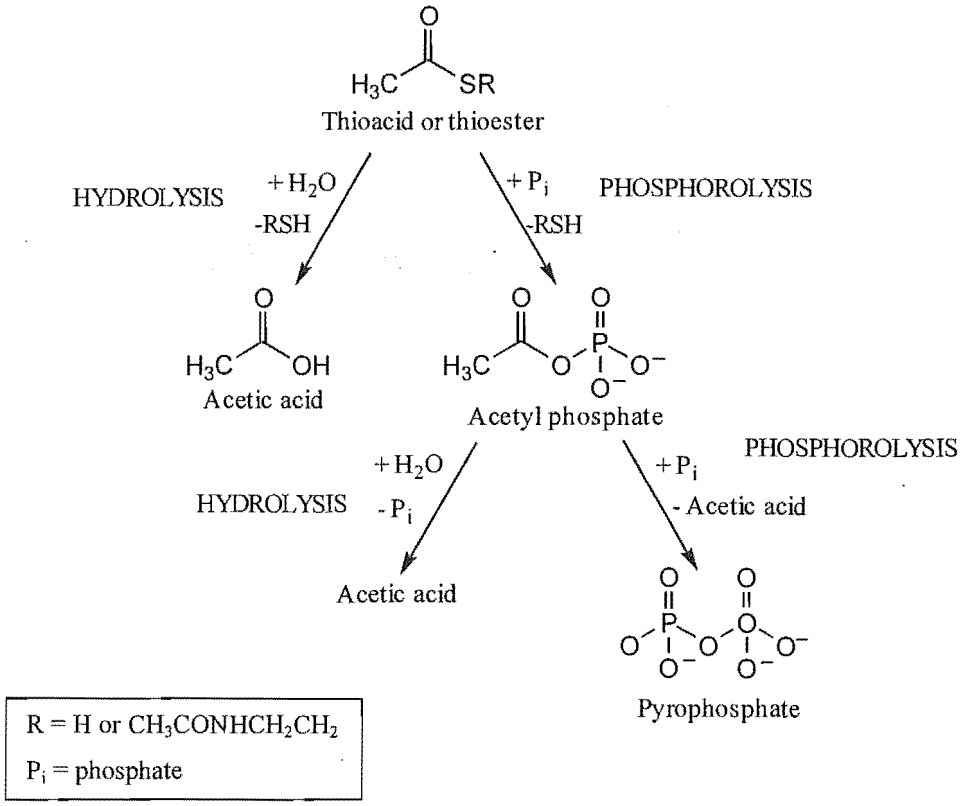


Figure 2.8: Competitive nucleophilic attack by phosphate and water

Nucleophilic attack by phosphate on the thioester or thioacid would generate acetyl phosphate; this reaction would compete with nucleophilic attack by water (which would result in acetic acid formation). Any acetyl phosphate that was formed would react quickly; nucleophilic attack by phosphate on this species (to give pyrophosphate) would again compete with attack by water (to give acetic acid).

2.2.3 Reaction mixture stoichiometries

It has been shown that in the reaction of acetyl phosphate with phosphate an excess of ferrous ions over phosphate ions is necessary for efficient pyrophosphate production to occur[14]. This ratio is thought to favour a ferrous-phosphate/ferrous-acetyl phosphate coprecipitate, because if ferrous ion concentrations are limiting phosphate ions will be selectively precipitated ahead of acetyl phosphate.

In these experiments it was also found that ferrous sulfide/ferrous phosphate coprecipitates were less efficient catalysts in the pyrophosphate-forming reaction than ferrous phosphate precipitates.

Both of these results influenced the design of the experiments reported below, and are discussed further where relevant.

2.2.4 Methods of analysis

NMR was used to determine the extent of thioester reaction and pyrophosphate production in reaction mixtures. ^{31}P NMR allowed a quick and easy assay for all phosphate species present in reaction mixtures, including phosphate and pyrophosphate (acetyl phosphate is a reactive and relatively short-lived species, and so, not surprisingly, it was not detected in any of the trials); and ^{1}H NMR was used to assess how much thioester or thioacid had reacted, with thioacetic acid, *N,S*-diacetyl cysteamine, *N*-acetyl cysteamine and acetic acid all giving isolated and easily identifiable CH_3 signals.

An obvious limitation in the interpretation of ^{1}H NMR data is that the relative losses of thioester or thioacid (as determined by acetic acid concentrations) through hydrolysis versus phosphorolysis cannot be ascertained (see figure 2.8).

2.2.5 Assessing recovery of pyrophosphate species

Before beginning pyrophosphate production experiments, it was necessary to first ensure that phosphate and pyrophosphate could be quantitatively recovered from the reaction mixtures. This was made difficult by the fact that both phosphate and

pyrophosphate will form insoluble precipitates with ferrous ions. It was therefore necessary to liberate the phosphate species from the precipitate before reaction mixtures were analysed by ^{31}P NMR. To do this, potassium cyanide was added to reaction mixtures at the end of the incubation period. Cyanide ions form stable complexes with iron ions, thereby liberating metal-bound phosphate species.

The extent of pyrophosphate recovery after the addition of cyanide ions was quantified in a simple experiment. Reaction solutions were directly spiked with small amounts of pyrophosphate ions before incubation. Thioacid trials were spiked with pyrophosphate so that 2% of all the phosphate centres in reaction mixtures were present as pyrophosphate, and thioester trials were spiked such that pyrophosphate represented 10% of the total phosphorous in solution. Solutions were incubated at 55°C for 10 days, with aliquots being taken intermittently, worked up with potassium cyanide, and analysed by ^{31}P NMR. Results are shown in figure 2.9.

It can be seen from the results shown in graph that pyrophosphate is relatively stable and able to be detected over several days under the reaction conditions being used. The steady decrease in pyrophosphate recovery probably reflects loss through hydrolysis.

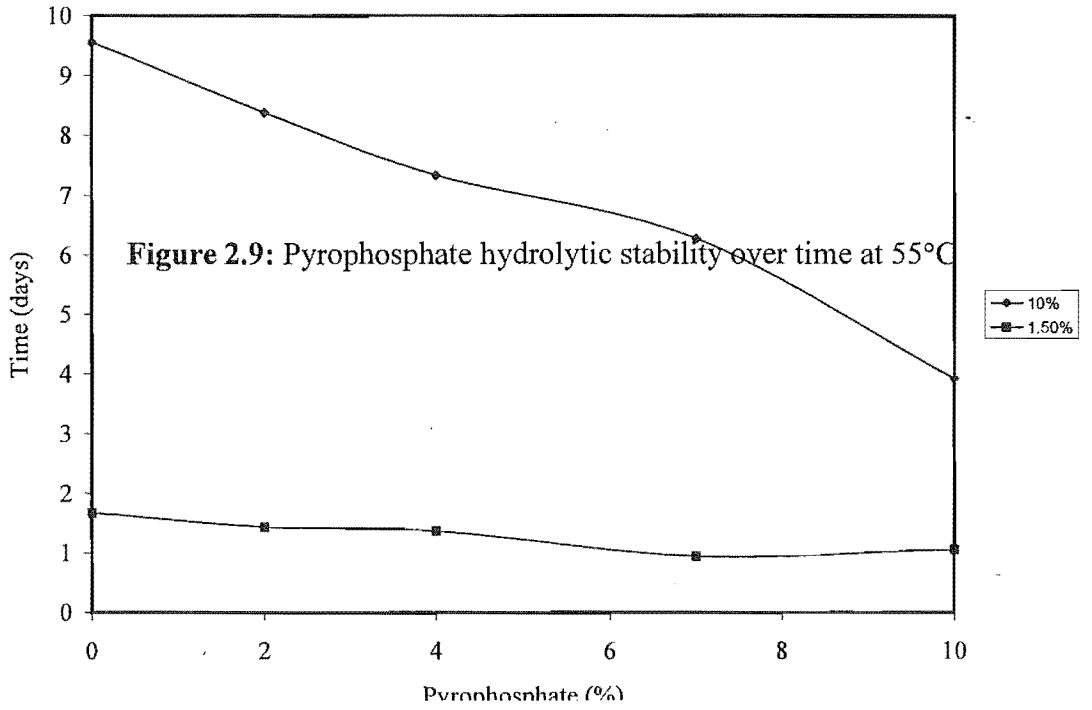


Figure 2.9: Pyrophosphate hydrolytic stability over time at 55°C

2.3 Production of pyrophosphate from acetyl phosphate

A reaction of acetyl phosphate and phosphate in the presence of ferrous ions was undertaken to confirm the veracity of de Zwart's findings. An iron:phosphate:acetyl phosphate ratio of 8:7:2 was used. A 5mL solution buffered to a pH of 6.5 and containing ferrous ions, acetyl phosphate and phosphate ions was generated; the acetyl phosphate and phosphate ions were added to the ferrous ions simultaneously to encourage coprecipitation. A white precipitate formed immediately, and the reaction mixture was incubated at 40°C for 24hr. ^{31}P NMR analysis showed that all of the acetyl phosphate had reacted to give pyrophosphate in a 17% yield; detectable pyrophosphate production from acetyl phosphate had been confirmed.

In the wake of these successful preliminary experiments, further studies were undertaken involving attempted pyrophosphate production with *N,S*-diacetyl cysteamine as a condensing agent.

2.4 *N,S*-Diacetyl cysteamine as condensing agent in attempted pyrophosphate production

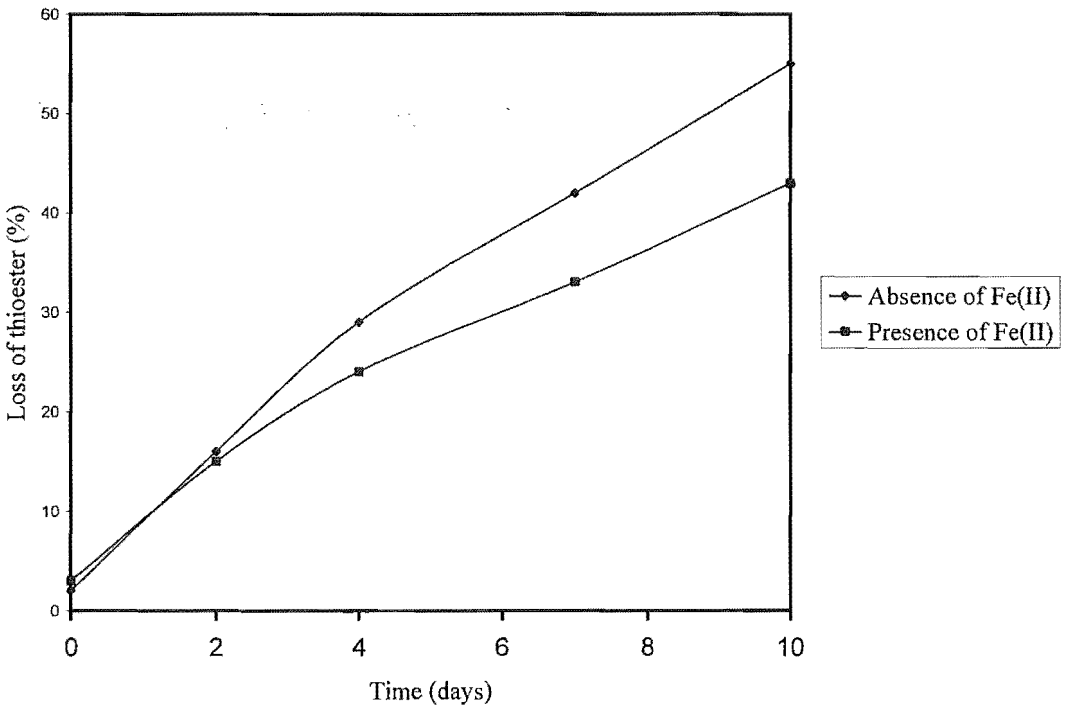
2.4.1 Catalysis by $FePO_4$/FeS coprecipitates

It had been shown by de Zwart that $FePO_4$/FeS coprecipitates catalyse the formation of pyrophosphate from acetyl phosphate and phosphate. FeS/$FePO_4$ coprecipitates were chosen over $FePO_4$ precipitates alone for these experiments in the hope that the sulfur component of the precipitate would encourage sulfur-based interaction with the thioester.

As was mentioned previously (in section 1.2.3), de Zwart has shown that reaction stoichiometries are critical. Despite his findings that high concentrations of phosphate ions (relative to ferrous ions) inhibit pyrophosphate formation from acetyl phosphate, the first set of experiments that I ran contained a relatively high concentration of phosphate ions; this was done in an attempt to encourage phosphorylitic attack on the thioester. It was hoped that any acetyl phosphate generated *in situ* on the precipitate

surface would remain precipitate-bound and participate in a second phosphoryl transfer reaction to give pyrophosphate.

A solution containing ferrous ions (25mM), sulfide ions (20mM), phosphate ions (160mM) and *N,S*-diacetyl cysteamine (100mM) was incubated at 55°C (reaction 18 in table 5.1). A control experiment without ferrous ions (and therefore without precipitates) was run in parallel (reaction 17 in table 5.1). Aliquots were taken at specified times, worked up by addition of cyanide followed by centrifugation, and analysed by ^{1}H and ^{31}P NMR. No pyrophosphate was detected in any of the solutions, but, interestingly, the ^{1}H NMR results showed that the presence of the precipitate in ferrous-ion containing reactions protected the thioester from hydrolysis (figure 2.10).



The most likely explanation for this result is that the thioester binds to the precipitate surface and in doing so is sequestered away from the solvent.

Figure 2.10: Loss of *N,S*-diacetyl cysteamine in the presence or absence of ferrous ions

It was possible that any acetyl phosphate being generated *in situ* might not precipitate efficiently in competition with such high relative concentrations of phosphate, and hence not be able to undergo further precipitate-mediated reactions. A second experiment was therefore run involving lower concentrations of phosphate ions. Solutions containing ferrous ions (85mM), sulfide ions (100mM), phosphate ions (60mM) and *N,S*-diacetyl cysteamine (400mM) were generated. Several experiments involving these stoichiometries were run, with the difference between them being the order of addition of reagents during the generation of reaction solutions. This was done in an attempt to encourage phosphorolytic attack upon the thioester by generating different types of coprecipitates. Reaction mixtures were incubated at 55°C for 10 days (reactions 8, 9 and 10 in table 5.1) and ^{31}P NMR analysis showed that little or no pyrophosphate production had occurred.

The ability of thioesters to act as condensing agents with $FeS/FePO_4$ coprecipitates had not been demonstrated under these experimental conditions. It was decided to investigate possible catalysis under conditions more closely related to those in the Weber experiment. In particular, the addition of imidazole to reaction mixtures was investigated.

2.4.2 Weber-type experiments involving imidazole

As described in section 1.1, Weber has reported formation of pyrophosphate from reaction mixtures containing *N,S*-diacetyl cysteamine, phosphate ions, hydroxyapatite and imidazole. To investigate any possible catalytic participation by imidazole in ferrous ion-mediated pyrophosphate formation trials, an experiment analogous to that reported in the Weber paper was run, with ferrous phosphate being used in place of hydroxyapatite. A mixture buffered to a pH of 6.5 and containing ferrous ions (130mM), phosphate ions (250mM), *N,S*-diacetyl cysteamine (100mM) and imidazole (200mM) was stirred at room temperature. An aliquot taken after 4 days and analysed by ^{31}P and ^{1}H NMR showed no pyrophosphate production and only a 14% loss of thioester; Weber reported a 60% loss of thioester with 8.2% pyrophosphate production after the same amount of time in his analogous hydroxyapatite mixtures. When my reaction mixture was analysed again after 11 days only 26% of the thioester had reacted, and no still pyrophosphate was detected.

Because of the slow rate of reaction of the thioester at room temperature, these experiments were repeated at a temperature of 65°C. After 17hr an aliquot was analysed by ^{31}P and ^{1}H NMR. All of the thioester had reacted but no pyrophosphate was detected. It was possible that the absence of pyrophosphate in these high temperature experiments reflects the poor hydrolytic stability of acetyl phosphate. It has been shown that at a temperature of 65°C the rate of hydrolysis of acetyl phosphate is significantly higher than the rate of phosphorolysis in the presence of $FePO_4$[14].

Rather than spending more time trying to optimise the temperature for reactions involving imidazole and ferrous phosphate, attempts were instead made to replicate Weber's experimental results. His reported protocol was followed closely, with the main exception being that the analysis of reaction mixtures was undertaken by ^{31}P NMR rather than radioactivity measurements of ^{32}P species; hence, radioactive [^{32}P] phosphate-containing molecules were not used. Because of this modified methodology reactions were carried out in volumes two orders of magnitudes higher than in his experiments (1.25mL instead of 12.5μL). A reaction mixture at a pH of 7.0 and containing phosphate ions (80mM), *N,S*-diacetyl cysteamine (100mM), imidazole (200mM) and hydroxyapatite (85mg in the 1.25mL reaction mixture) was stirred at room temperature for 6 days. EDTA was used to displace phosphate species from the precipitate during sample workup, as had been reported in the Weber paper. Pyrophosphate could not be detected by ^{31}P, and ^{1}H NMR analysis showed that only 5% of the thioester had reacted. The experiment was repeated at a temperature of 50°C. After 6 days 34% of the thioester had reacted, but no pyrophosphate could be detected. Weber reported just under 10% pyrophosphate production (based on thioester consumption) after a similar amount of thioester reaction. I was therefore unable to replicate Weber's results; the thioester was reacting more slowly than what had been reported, and when it was made to react, through the use of higher incubation temperatures, it did not act as a condensing agent in pyrophosphate production.

In view of this disappointing result it was decided to discontinue thioester studies in preference for experiments involving thioacids as possible condensing agents.

2.5 Attempted pyrophosphate production using thioacid

Attention was next given to reaction systems involving ferrous sulfide/phosphate coprecipitates and thioacetic acid. As mentioned in section 2.1, it was hoped that thioacetic acid would interact with the precipitate through the thioacid functionality, and that this interaction would facilitate phosphorolysis by coprecipitated phosphate.

Many trials were run, varying the reagent stoichiometries, addition of reagents during the generation of reaction mixtures, and incubation times and temperatures. Results are summarised in table 5.7, and the three most significant sets of experiments are discussed in more detail below.

Reaction mixtures containing similar amounts of ferrous ions, sulfide ions and phosphate ions and a ten-fold molar excess of thioacetic acid were generated. The black FeS precipitate appeared to be present at much higher levels than the white $FePO_4$ precipitate. Reaction mixtures were incubated for 8 days at 50°C (reaction 4 in table 5.7), with aliquots being taken intermittently and analysed by ^{1}H and ^{31}P NMR; no pyrophosphate was detected, and about half of the thioester had reacted. An interesting observation, however, was that the black FeS precipitate stayed in suspension longer after shaking reaction vials than in analogous thioester trials. This presumably indicated a higher FeS precipitate surface area:volume in thioacid trials; which suggests that the thioacid was interacting with the FeS precipitate in a way that was not possible for the thioester. This was considered an encouraging result.

Given that de Zwart had shown that sulfide ions inhibit the ability of $FePO_4$ to catalyse the production of pyrophosphate from acetyl phosphate, further experiments were undertaken without added sulfide ions. Mixtures containing equimolar amounts of ferrous ions and phosphate ions with a six-fold molar excess of thioacetic acid were incubated at 50°C for 8 days (reaction 5 in table 5.3), with aliquots being taken intermittently and analysed by ^{1}H and ^{31}P NMR. After 8 days 55% of the thioacid had reacted, but no pyrophosphate had been detected.

In an attempt to encourage phosphorylitic attack of the thioacid, a reaction was run in the presence of a relatively high concentration of phosphate ions. A reaction mixture

containing ferrous ions, sulfide ions, thioacetic acid and a relatively high concentration of phosphate ions was generated (reaction 16 in table 5.3). Sulfide ions were added to a solution of ferrous ions and thioacetic acid, with a black FeS precipitate forming immediately. Phosphate ions were next added, and whilst a few white flecks of $FePO_4$ could be seen in the reaction mixture, the black FeS precipitate dominated. A control solution without ferrous ions (and therefore without precipitates) was also generated. Reaction mixtures were incubated at 55°C. When the first aliquot was taken after 2 days, it was observed that a white $FePO_4$ precipitate had all but completely replaced the black FeS precipitate in the ferrous ion-containing experiment. This phenomenon was not seen in the above reactions involving lower relative concentrations of phosphate ions, nor in analogous experiments where *N,S*-diacetyl cysteamine was used in place of thioacetic acid. This was an encouraging result, as it suggested that thioacetic acid (presumably precipitate-bound) encouraged the interaction of phosphate ions with the FeS precipitate. ^{1}H NMR analysis showed that the thioacid was reacting more slowly; however. After 10 days only approximately 40% of the thioacid had reacted in the presence and absence of ferrous ions. This rate of reaction was much lower than in the above two experiments. This could have been due to either different rates of hydrolysis of thioacetic acid in the presence of FeS or $FePO_4$, or, more likely, the absence of free ferrous ions in solution in this latter trial due to high concentrations of phosphate. No pyrophosphate was detected in reaction mixtures.

It was disappointing to not detect pyrophosphate production in any of the trials, but some interesting results regarding thioacetic acid hydrolysis in the presence of ferrous precipitates had been obtained. It was decided to further investigate the effect of free ferrous ions, $FePO_4$ precipitates and FeS precipitates on thioacetic acid hydrolysis.

2.6 Thioacetic acid hydrolysis

In order to further explore the effects of FeS and $FePO_4$ precipitates on thioacetic acid hydrolysis, the rate of the conversion of thioacetic acid to acetic acid in the presence or absence of these precipitates was monitored by ^{1}H NMR. A mixture containing equimolar amounts of ferrous ions and thioacetic acid was generated. To an aliquot of

this solution was added a three-fold molar excess of sulfide ions; the characteristic black FeS precipitate formed immediately. To a second aliquot of the ferrous ion/thioacetic acid solution was added a three-fold molar excess of phosphate ions; the white $FePO_4$ precipitate formed immediately. Control solutions without added ferrous ions and/or sulfide ions and phosphate ions were also generated. Reaction mixtures were incubated at 65°C for 17hr. At the end of the incubation period reaction mixtures were worked up and analysed by ^1H NMR. A comparison of thioacetic acid and acetic acid peak areas was used to calculate the loss of thioacetic acid; results are summarised in table 2.1.

Nature of Fe(II) species	% loss of thioacetic acid
None	40
Free ferrous ions	90
$FePO_4$	66
FeS	35

Table 2.1: Hydrolysis of thioacetic acid in the presence of Fe^{2+}, $FePO_4$ or FeS

It can be seen that dissolved ferrous ions catalyse the hydrolysis of thioacetic acid. This catalysis could be due to ferrous ions withdrawing electron density away from bound thioacids activating them for nucleophilic attack, in conjunction with attack by coordinated water.

The FeS and $FePO_4$ precipitates do not catalyse the hydrolysis of thioacetic acid to the same extent as free ferrous ions in solution. Interestingly, the FeS precipitate does not catalyse hydrolysis at all, whereas the $FePO_4$ precipitate does. $FePO_4$ is less soluble than FeS so the higher rates of hydrolysis in $FePO_4$ experiments is probably not due to free ferrous ions in solution. Rather, the $FePO_4$ precipitate itself will be catalysing the thioacid hydrolysis. This catalysis presumably occurs by Fe(II) centres on the precipitate surface catalysing the hydrolysis of bound thioacetic by coordinated water molecules. The possible appearance of acetic acid in this experiment by

phosphorolytic attack on thioacetic acid to give acetyl phosphate, followed by hydrolysis of this species (to give acetic acid) is unlikely because no pyrophosphate was detected by ^{31}P NMR, suggesting that acetyl phosphate production was minimal.

In light of the observations described in the previous section that suggest that thioacetic acid interacts with FeS precipitates, it was surprising to see no increase in rates of hydrolysis when thioacetic acid was incubated with FeS (this result was checked and confirmed several times). The reason for this result is not known, but may reflect unfavourable orientations of bound water molecules relative to thioacid molecules on the precipitate surface.

2.7 Conclusions

In summary, then, neither *N,S*-diacetyl cysteamine nor thioacetic acid was shown to be effective an dehydrating agent in the production of pyrophosphate over ferrous sulfide and/or ferrous phosphate precipitates. It was shown that ferrous phosphate catalyses the formation of pyrophosphate from acetyl phosphate; from this it follows that the lack of demonstrable pyrophosphate production in my trials was probably due to insufficiently low levels of acetyl phosphate formation.

Results were presented that showed that free ferrous ions catalyse the hydrolysis of thioacetic acid. $FePO_4$ precipitates also show this catalysis (at a slower rate), but, surprisingly, FeS does not. Observations were made that strongly suggested that thioacetic acid interacts with FeS precipitates, so differences in coordinative chemistry between FeS and $FePO_4$ precipitates are presumably responsible for the differences in catalysis.

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Chapter 3 Redox chemistry

3.1 Introduction

Redox reactions are of great significance in metabolic systems, being at the heart of both biological energy generation and biosynthesis. Thioesters are involved as substrates and products in many of these reactions (see section 1.3.1), and protein-bound iron-sulfur clusters act as ultimate donors or acceptors of such electrons (see section 1.3.2). Investigation of biomimetic systems involving thioesters and ferrous and sulfide ions therefore has the potential to shed light on fundamental biochemistry, and is of particular interest with respect to the origins of life.

Just as in metabolism (see section 1.3.1 and 1.3.2), both the ferrous/ferric ion [1-3] and sulfide/disulfide [4-6] redox couples have been shown to operate in biomimetic redox chemistry, and thioesters have been involved as substrates [1, 2] and products in several plausibly prebiotic redox processes [7, 8]. This chapter describes investigation of the redox chemistry of thioesters and an analogous thioacid mediated by ferrous and sulfide ions.

3.2 Attempted reduction of *N,S*-diacetyl cysteamine by ferrous ions

This section describes an investigation into ferrous ion-mediated redox chemistry of *N,S*-diacetyl cysteamine. *N,S*-Diacetyl cysteamine was initially chosen as a substrate because, as was mentioned in section 2.2.1, it effectively represents the reactive functional group of acetyl-CoA, an important thioester in biochemistry. *N,S*-Diacetyl cysteamine was synthesised in a single step by the acetylation of cysteamine with acetic anhydride, as described in section 2.2.1 (see figure 2.7).

The redox reactions investigated involved the anaerobic incubation of solutions buffered to a pH of 6.8 and containing *N,S*-diacetyl cysteamine and ferrous ions at 45°C for 24 hours. ^{1}H NMR was used to assess the extent of loss of *N,S*-diacetyl cysteamine and appearance of *N*-acetyl cysteamine (see section 2.2.3 for details of ^{1}H

NMR analysis), and TLC was used to assay for acetaldehyde, a likely initial product of *N,S*-diacetyl cysteamine reduction (figure 3.1).

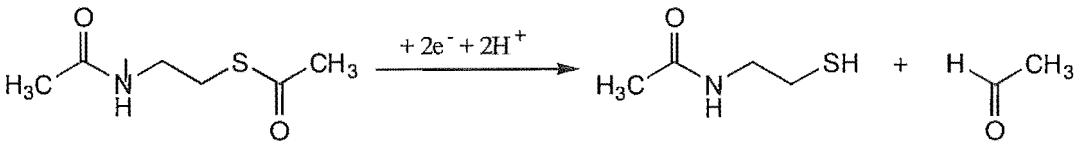

Figure 3.1: reduction of *N,S*-diacetyl cysteamine

Analysis of acetaldehyde itself was made difficult by its low boiling point (40°C), and so acetaldehyde 2,4-dinitrophenyl hydrazone, the product of the reaction between acetaldehyde and 2,4-dinitrophenyl hydrazine, was generated *in situ* and assayed for. Acetaldehyde hydrazone is a brightly coloured orange complex of low volatility, making it an ideal compound for TLC analysis. After incubation, 2,4-DNP was directly added to the reaction solutions, which were then extracted with ether. The ether extracts were analysed by TLC, with the hydrazone and the unreacted hydrazine giving clear and distinct signals. This allowed reactions to be qualitatively monitored via the production of the hydrazone.

Interpretation of the 2,4-DNP/TLC assay results was complicated by the regular appearance, during analysis of control experiments run in the absence of *N,S*-diacetyl cysteamine, of faint orange spots on the TLC plates with an R_f value similar to that for acetaldehyde hydrazone. It was discovered that this was due to contamination arising *de novo* in the 2,4-DNP solution. For this reason, freshly made solutions were used for all analyses, which minimised, but did not eliminate, the problem of contamination.

The assay was shown be capable of detecting acetaldehyde in aqueous solutions down to a concentration of $1x10^{-4}$M; whereas the minimum level of detectable recovery from reaction mixtures spiked with acetaldehyde and incubated under the specified reaction conditions was $5x10^{-4}$M. The difference between these values shows that

some loss of acetaldehyde was occurring during incubation, possibly through aldol-condensations, volatilisation, and/or redox chemistry. This detection limit for acetaldehyde of $5x10^{-4}$ in reaction solutions corresponded to 5% of the *N,S*-diacetyl cysteamine that was used in each trial. An obvious limitation to this assay, therefore, was its low sensitivity; it was, however, deemed appropriate for an initial investigation of the redox chemistry of *N,S*-diacetyl cysteamine.

Reaction mixtures were generated and incubated anaerobically. Comparison of *N,S*-diacetyl cysteamine and *N*-acetyl cysteamine peak areas from ^{1}H NMR data showed that about half of the thioester had reacted. Acetaldehyde was not detected in these trials; that is, no acetaldehyde hydrazone spot was seen in the TLC assays. All that can therefore be concluded is that under the specified reaction conditions, approximately 50% of the thioester reacted, and acetaldehyde, if present, was produced in less than 10% yield (based on loss of thioester). It is impossible to draw more precise conclusions regarding the reduction of thioesters by ferrous ions from these results. The relative insensitivity of the assay has already been discussed, and more sensitive assays were not readily available. Further investigation with this system was therefore abandoned in favour of investigating a system involving chromophore-containing molecules, which could be analysed more rigorously.

3.3 Attempted reduction of *N,S*-dibenzoyl cysteamine by ferrous ions

N,S-Dibenzoyl cysteamine was chosen as the next thioester to be used as a substrate in redox trials. It was chosen in place of the corresponding diacetyl compound because the benzene chromophores allowed a variety of analytical methods to be used to assay the reactions. Figure 3.2 shows some of the benzene-containing compounds that may be produced when this thioester is incubated in an aqueous solution with a reducing agent.

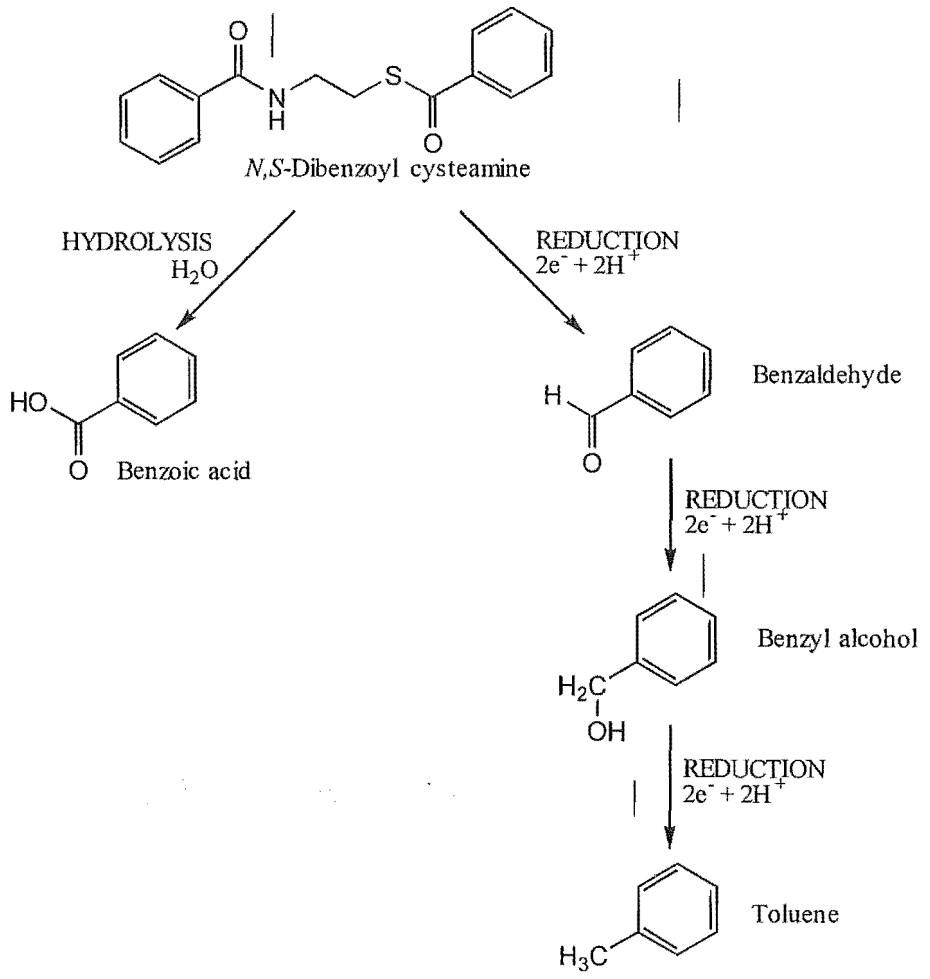

Figure 3.2: Some of the compounds that may be present in reaction solutions

Aurther advantage of using the dibenzoyl rather than the diacetyl cysteamine derivative is that benzaldehyde, a likely reduction product of *N,S*-dibenzoyl cysteamine, is not as volatile as acetaldehyde, nor is it capable of aldol condensations, due to the absence of an acidic α-H. Not surprisingly, detection limits of benzaldehyde using TLC with a UV lamp were also shown to be significantly lower than in the acetaldehyde trials using TLC and 2,4-DNP. A significant disadvantage in using the dibenzoyl- rather than the diacetyl-cysteamine derivative, however, lay in the hydrophobicity of benzene groups. The solubility of *N,S*-dibenzoyl cysteamine in water was found to be very low, and reactions were therefore run in 60% methanol solutions. This not only compromised the biomimetic nature of the trials, it also caused problems with buffering; PIPES, MES and HEPES buffers were not soluble in

this solvent. Reactions were therefore not buffered, and were simply left at a pH of around 5.

N,S-Dibenzoyl cysteamine was synthesised by the benzoylation of cysteamine using benzoyl chloride (figure 3.3) in a protocol based on that reported by Green *et al*[9].

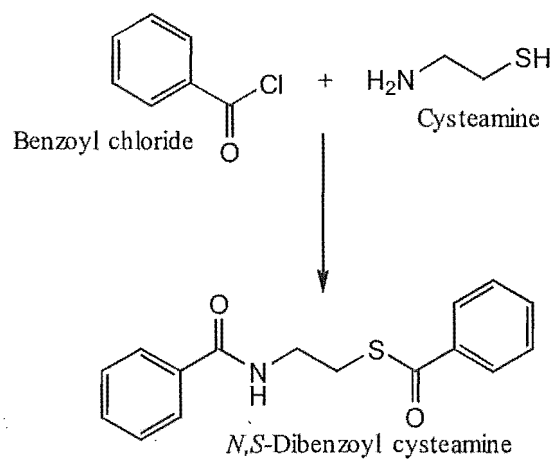

Figure 3.3: The synthesis of *N,S*-dibenzoyl cysteamine by the benzoylation of cysteamine with benzoyl chloride

Reaction solutions containing *N,S*-dibenzoyl cysteamine and ferrous ions in 60% methanol were generated and incubated anaerobically for 48hrs at 45°C. Experiments without ferrous ions were run in parallel. Reaction solutions were assayed using TLC, with visualisation of species by UV lamp and 2,4-DNP spray. Numerous spots were seen under the UV lamp, two of which were shown, by running reference samples in parallel, to be benzoic acid and the thioester starting compound. A third spot migrated at a similar rate to benzaldehyde and gave an orange colour when sprayed with 2,4-DNP. This result, however, was also seen in some trials run in the absence of ferrous ions, and so could not be taken as a reliable indicator of ferrous ion-mediated thioester reduction.

In order to investigate this promising result further, it was decided to develop a quantitative assay based on HPLC that would give information on the extent of

thioester reaction and the relative ratios of the various products. To facilitate these experiments, two other experimental details were also changed; firstly, the unnecessary hydrophobicity of the *N*-acyl group was addressed; and, secondly, the more powerful reductant, FeS, was used in place of ferrous ions alone.

3.4 Attempted reduction of *N*-acetyl, *S*-benzoyl cysteamine by FeS

This section describes attempts to detect reduction of *N*-acetyl, *S*-benzoyl cysteamine by FeS. *N*-Acetyl, *S*-benzoyl cysteamine was chosen as the next cysteamine-derived thioester for investigation because of the combination of the *S*-benzoyl chromophore (to allow UV-based analyses) with the more hydrophilic *N*-acetyl group (to improve water solubility). Indeed, *N*-acetyl, *S*-benzoyl cysteamine was found to be more soluble in water than *N,S*-dibenzoyl cysteamine, although it's solubility was still relatively low. Reactions were therefore carried out in aqueous solutions containing 10% acetonitrile by volume.

N-Acetyl, *S*-benzoyl cysteamine was synthesised by the partial hydrolysis of *N,S*-diacetyl cysteamine with potassium hydroxide, as reported by Schwab and Klassen[10], followed by benzoylation of the resulting *N*-acetyl cysteamine with benzoyl chloride, using a protocol based on that reported by Green *et al*[9] (figure 3.4).

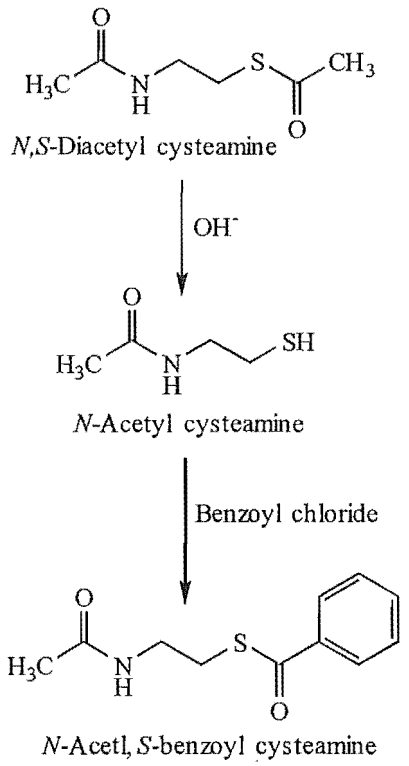

Figure 3.4: Synthesis of *N*-acetyl, *S*-benzoyl cysteamine

Ferrous sulfide was chosen as the reductant in these experiments over ferrous ions alone because it is a stronger reducing agent, capable of both sulfide/disulfide and Fe(II)/Fe(III) redox chemistry (figure 3.5). The higher reductive power of the FeS/H_2S system over ferrous ions can be explained in part by the fact that FeS_2, or pyrite, is a stable crystalline solid, and its oxidative formation from ferrous sulfide provides a thermodynamic driving force for redox reactions. Several interesting and potentially prebiotic redox reactions involving the FeS/H_2S reducing agent have already been demonstrated [7, 11, 12].

$$Fe^{2+} \rightarrow Fe^{3+} + e^- \quad E^0 = -620mV$$

$$FeS + HS^- \rightarrow FeS_2 + H^+ + 2e^- \quad E^0 = -770mV$$

Figure 3.5: Standard oxidation potentials of Fe(II) ions and FeS

Reaction mixtures buffered to a pH of 6.8 and containing *N*-acetyl, *S*-benzoyl cysteamine, ferrous sulfide and free sulfide ions in 5% acetonitrile solutions were incubated for 5 days at 65°C. Control experiments without ferrous ions were run in parallel. Reaction mixtures were to be analysed by HPLC, using a protocol that had been shown (by using authentic samples) to separate *N*-acetyl, *S*-benzoyl cysteamine, benzoic acid, thiobenzoic acid, benzaldehyde, benzyl alcohol and toluene unambiguously for identification. Reaction mixtures were prepared for analysis by the addition of a 12-fold excess of KCN to displace metal-bound organics and remove any iron ions from solution before HPLC analysis. This was important because iron complexes had been shown to precipitate on C_{18} HPLC columns in the past, causing irregular flow rates and blockages. The resulting solutions were centrifuged and the supernatants were passed through a C_{18} cartridge, with the organics being eluted using acetonitrile. The resulting acetonitrile solutions were analysed by HPLC. A trace from a typical reaction run in the absence of ferrous ions is shown below (figure 3.6).

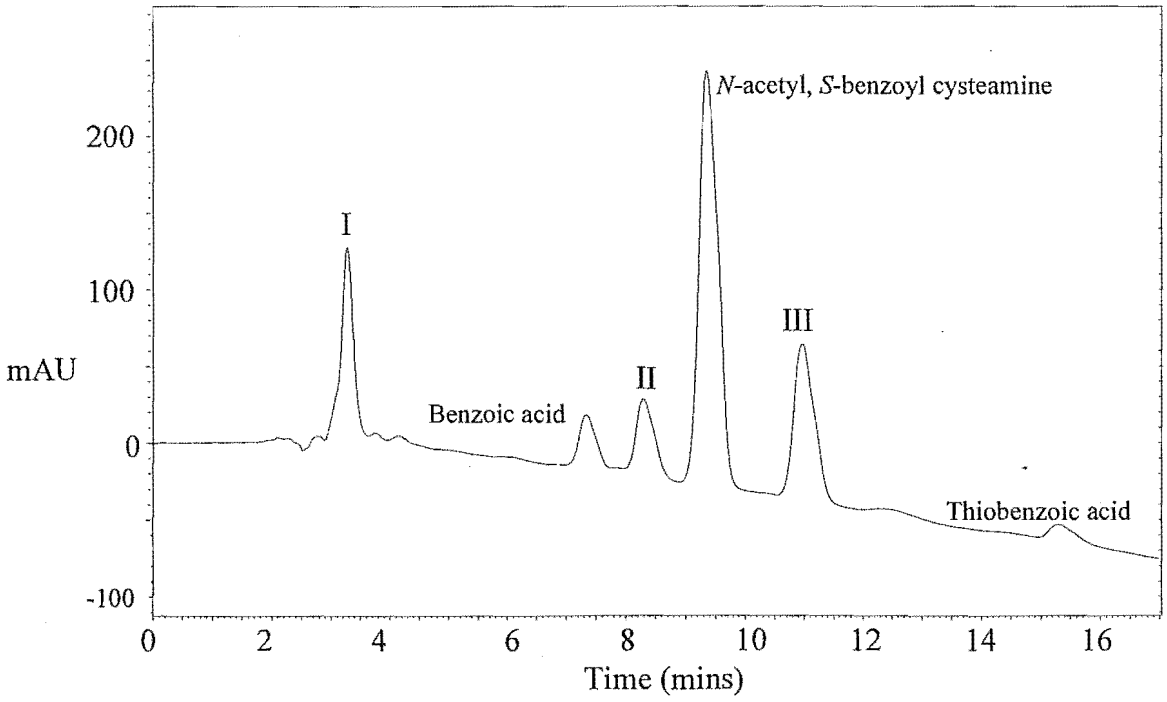

Figure 3.6: An HPLC trace obtained from a reaction mixture containing *N*-acetyl, *S*-benzoyl cysteamine and FeS

It will be assumed in the following discussion that each of the species corresponding to peaks in the HPLC traces absorbs UV light to the same extent. The relative concentrations of chromophore-containing compounds are therefore considered to be directly proportional to the relative peak areas in HPLC traces.

It could be deduced from HPLC traces that approximately 50% of the thioester reacted in the absence of ferrous ions, and around 75% reacted in their presence. No new peaks could be seen in the HPLC traces obtained from analysis of reactions run in the presence of ferrous ions. Six distinct peaks were seen in each of the HPLC traces; three of these were identified (by spiking with authentic samples) as *N*-acetyl, *S*-benzoyl cysteamine, benzoic acid and thiobenzoic acid. Benzoic acid is the product of thioester hydrolysis, and its rate of appearance increased in the presence of FeS. This rate of increase varied between trials; but usually fell between a factor of 1.5 and 2. The small amount of thiobenzoic acid (2.5%) which appeared in reaction mixtures incubated in the presence of sulfide ions (independent of the presence or absence of ferrous ions) was most likely the product of thioester thiolysis, although a mechanism for its appearance involving thioester reduction by sulfide ions cannot be excluded. The identity of the remaining three peaks in the HPLC traces is unknown; they are not benzaldehyde, benzyl alcohol or toluene, and their appearance in traces occurs independently of both the presence of ferrous ions and addition of cyanide during the workup. They therefore do not represent products of ferrous ion-mediated reduction, nor artefacts from cyanide-catalysed chemistry. Ferrous ions increased the rate of appearance of two of the three unknown compounds (labelled I and III) corresponding to these peaks.

In summary, the inclusion of ferrous sulfide in reaction mixtures containing *N*-acetyl, *S*-benzoyl cysteamine did not give rise to any new detectable compounds when the mixtures were analysed by HPLC. The rate of hydrolysis increased in the presence of FeS, as did the rate of appearance of two of the three unknown compounds (labelled I and III in figure 3.6) seen in the HPLC traces. As expected, a concomitant loss of the thioester was also detected when ferrous ions were included in experiments. Because all of the three unknown compounds appeared in reaction solutions run in the absence

of ferrous ions, they were not considered to be products of iron-sulfur-mediated redox chemistry. Further investigation into ferrous sulfide-mediated reactions of *N*-acetyl, *S*-benzoyl cysteamine was therefore abandoned in favour of investigating the analogous chemistry of thiobenzoic acid.

3.5 Attempted reduction of thiobenzoic acid

As has been discussed in section 1.2.4, metal-bound thioacids may have been involved in prebiotic chemistry, and could be the predecessors of thioesters found in contemporary metabolism. It was hoped in these experiments that the binding of thiobenzoic acid to ferrous ions on the FeS precipitate surface would facilitate reduction of the thioacid by bringing it in close proximity to the reductant. The reason for choosing thiobenzoic acid specifically as the thioacid to be used was that, as for the *S*-benzoyl thioesters, the benzene chromophore allowed analysis of reaction mixtures by HPLC. Another distinct advantage of using thiobenzoic acid was that, unlike the *S*-benzoyl thioesters, it is relatively soluble in aqueous solutions at a pH of 6.8.

Thiobenzoic acid was synthesised by the method of Noble *et. al.* [13], and involved the reaction of benzoyl chloride with sulfide ions in a 90% aqueous ethanol solvent (figure 3.7).

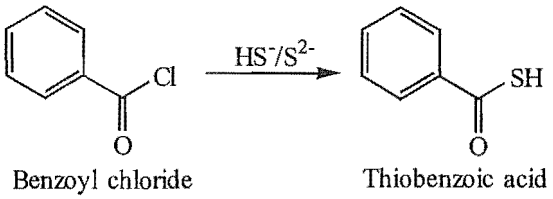

Figure 3.7: Synthesis of thiobenzoic acid

In an analogous fashion to the *N*-acetyl, *S*-benzoyl cysteamine trials, solutions buffered to a pH of 6.8 and containing thiobenzoic acid, ferrous sulfide and free

sulfide ions were incubated for 5 days at 65°C. Experiments that did not contain ferrous ions were run in parallel. The reactions were worked up as for the *N*-acetyl, *S*-benzoyl cysteamine trials and analysed by HPLC.

It could be immediately seen in the HPLC traces that two of the three unknown compounds arising in the *N*-acetyl, *S*-benzoyl experiments were also arising in the thiobenzoic acid experiments (corresponding to peaks II and III in figure 3.6). In an attempt to identify the unknown compounds, a reaction sample from a thiobenzoic acid experiment was run through an HPLC (with UV detector) and analysed on-line by MS using atmospheric pressure chemical ionisation. Both positive- and negative-ion mass spectrometry analyses detected one of the unknown compounds (corresponding to peak II in figure 3.6), but its structure could not be deduced from the mass spectrometry data. The size of the peak arising from this compound in HPLC traces was the same in trials run in the presence or absence of ferrous ions. Further attempts to identify the compound were therefore abandoned, because its appearance in reaction mixtures was not related to the presence of ferrous ions.

From the HPLC traces it could be seen that 30% of the thioacid reacts in the absence of ferrous ions, and 40% reacts in their presence, which is less reaction than had been observed in analogous reactions involving *N*-acetyl, *S*-benzoyl. This difference in the extent of reaction is probably best explained by the fact that thioesters are generally more reactive than thioacids; due, in part, to the anionic nature of thiocarboxylates and the fact that thiols are better leaving groups than free sulfide. As in the thioester experiments, ferrous sulfide increased the rate of hydrolysis of the thiobenzoic acid substrate. The rate of appearance of one of the two unknown compounds (corresponding to peak III in figure 3.6) also increased when ferrous ions were included in reaction mixtures.

3.6 Conclusion

Preliminary investigations into the ferrous ion-mediated redox chemistry of thioesters using *N,S*-diacetyl cysteamine as a substrate did not reveal any evidence of thioester reduction over the course of an incubation. The 2,4-DNP-based TLC assay could be

used to assay for only one of several possible reduction products, and was hindered by a relatively high detection limit.

Experiments involving *N,S*-dibenzoyl cysteamine were hindered by its low solubility in aqueous solutions. Analysis of reaction solutions using a UV-based TLC assay gave promising results, with the detection of a compound that migrated at a similar rate to benzaldehyde during TLC development and that gave an orange signal when TLC plates were sprayed with 2,4-DNP solution. This compound was also detected in trials run in the absence of ferrous ions, however, and so was not direct evidence of ferrous ion-mediated thioester reduction. It did, however, stimulate further investigation into thioester and thioacid reduction using more water-soluble substrates and the more powerful FeS/H_2S reducing agent, along with HPLC analysis to give accurate quantitative data.

After incubation with ferrous sulfide and excess sulfide ions, *N*-Acetyl, *S*-benzoyl cysteamine reacted at a higher rate than thiobenzoic acid, and the presence of ferrous sulfide was shown to increase the rate of reaction of both substrates. Benzoic acid, the product of thioester or thioacid hydrolysis, could be identified in both types of reaction mixtures, and the rate of its appearance increased in the presence of FeS. The appearance of thiobenzoic acid in thioester-containing reaction mixtures was detected, presumably as a result of thiolysis of the thioester. It was impossible to assess the extent of thiolysis in thiobenzoic acid trials because, of course, reactant and product thiobenzoic acid molecules were indistinguishable. Several peaks corresponding to unknown compounds were present in HPLC traces from both types of reaction mixtures, but all appeared independently of FeS, and so were not considered to be products of ferrous- and/or sulfide-mediated reduction. The rate of appearance of two of these three compounds increased in the presence of FeS. HPLC/MS analysis was used in an attempt to characterise these unknown reaction products. One of the unknown compounds was detected, but its structure could not be deduced from this information. No evidence for ferrous-sulfide-mediated reduction of *N*-acetyl, *S*-benzoyl cysteamine or thiobenzoic acid by ferrous sulfide was seen.

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Chapter 4 Amide bond formation

4.1 Introduction

Life as we know it is completely dependent on proteins, that is, linear chains of amino acid residues linked through peptide bonds. Peptides are today biosynthesised by two different mechanisms, ribosomal and non-ribosomal synthesis. Ribosomal peptide biosynthesis is a process ubiquitous to all life, and is used by cells to make most, if not all, cellular polypeptides. Non-ribosomal peptide biosynthesis on the other hand, first discovered in 1969 during investigations into the biosynthesis of gramicidin S[1], only occurs in bacteria and some eukarytic cells, and is responsible for the production of more than two hundred compounds[2, 3]. Non-ribosomal peptide biosynthesis is believed by some to be the more archaic of the two processes[4, 5], and involves the activation and correct orientation of amino acids as protein-bound thioesters. In non-ribosomal peptide biosynthesis, the amine group from an amino thioester attacks at the carbonyl carbon of an adjacent amino thioester to generate an amide bond (figure 4.1).

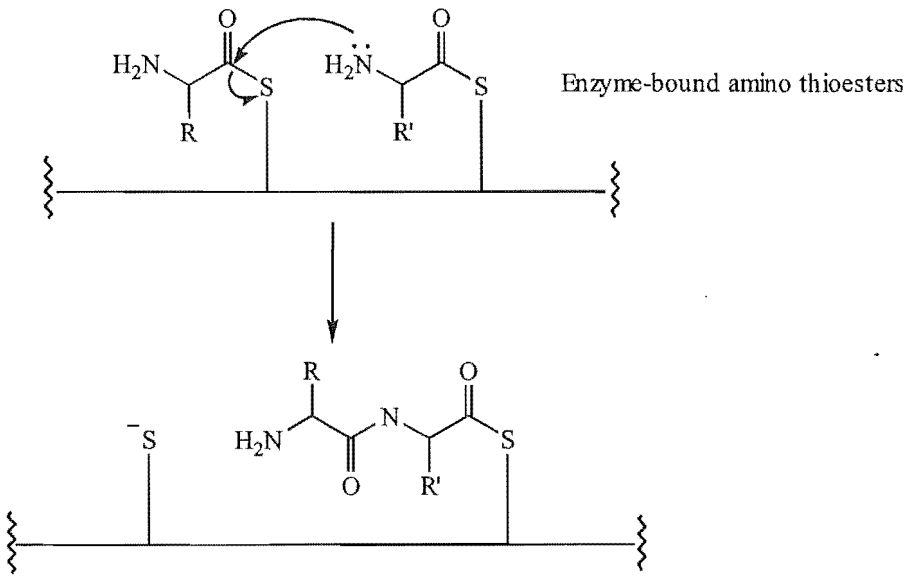

Figure 4.1: A schematic representation of non-ribosomal peptide biosynthesis

It has been postulated that the ancestral mechanism of biochemical peptide bond formation may have involved thioesters[6], or an equivalent species[7]. This draws support fom the observation that peptide bonds will form spontaneously in solutions of amines and thioesters[8, 9]. The potentially prebiotic formation of peptide bonds by a mechanism possibly related to that involving thioesters has been demonstrated by Huber and Wächtershäuser[10], as part of a paper on prebiotic carbon fixation, introduced in section 1.2.4. The authors reported production of a thioester, CH_3COSCH_3, in a reducing reaction mixture containing carbon monoxide, methane thiol and an (Fe, Ni)S coprecipitate (figure 4.2, left hand branch). This system can be considered a mimic of the biological carbon monoxide dehydrogenase/acetyl-CoA synthetase-catalysed reaction[11]. Whilst the plausibly prebiotic formation of thioesters is an interesting result in itself, the remainder of this discussion is concerned with a second observation made by the investigators. They discovered that in the presence of aniline acetanilide was formed, and suggested the following reaction mechanism, involving metal-bound thioacids (figure 4.4, right hand branch).

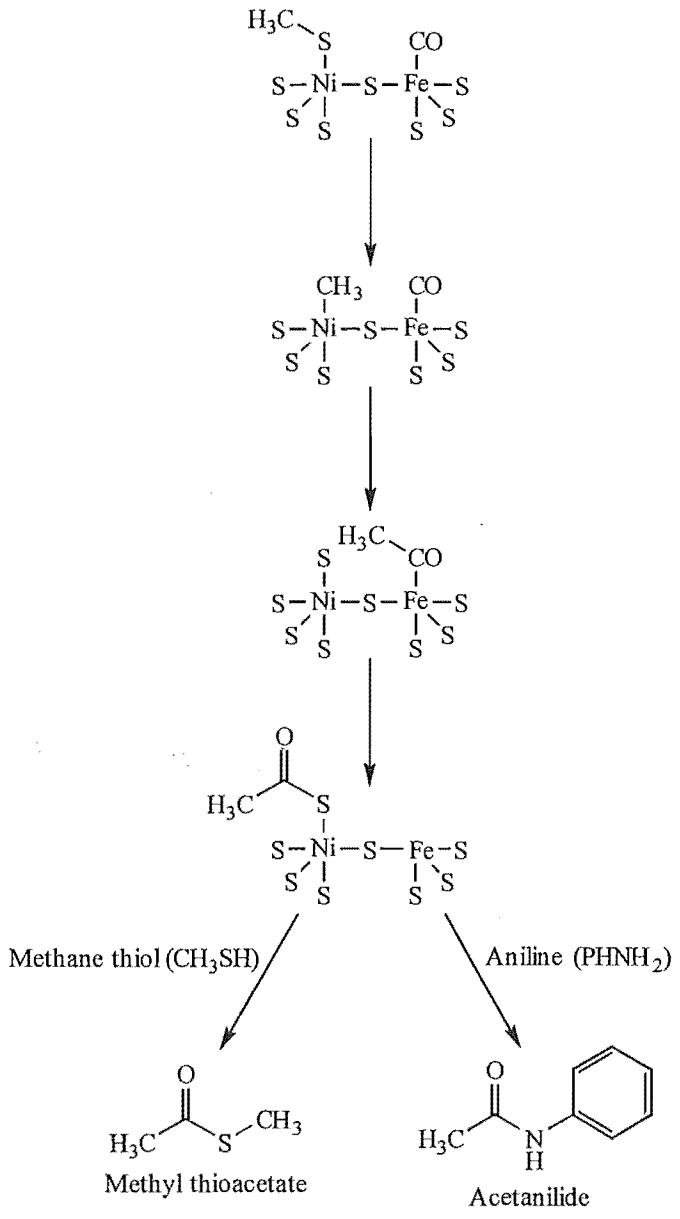

Figure 4.2: Postulated reaction mechanism from the Huber-Wachtershauser paper

As was discussed in section 1.2.4, metal-bound thioacids can be thought of as thioester analogues, and may have preceded them in metabolism. The right-hand branch of the above process (interception of the hypothesised thioacid intermediate by an amine nucleophile) is therefore a biomimetic analogue of non-ribosomal peptide biosynthesis, with the metal sulfide precipitate fulfilling both the catalytic and template functions of today's biosynthetic enzymes.

This chapter describes experiments designed to assess the possible roles of iron, nickel and sulfur in the plausibly prebiotic acylation of amines by thioacids.

4.2 The role of iron in amide bond formation

Huber and Wächtershäuser's mechanistic proposal has stimulated further investigation into possible ferrous ion-catalysis of amide bond formation using thioacids and amine nucleophiles. Meade[12] was able to demonstrate that ferrous ions increase the efficiency of acylation of alanine and glycine by thioacetic acid, and Hampton[13] went on to demonstrate an increase in the rate of amide bond formation between Z-amino thioacids and amino acids or amino acid methyl esters in the presence of ferrous ions. A catalytic mechanism involving ferrous ion coordination chemistry was proposed as one possible basis for this catalysis (figure 4.3).

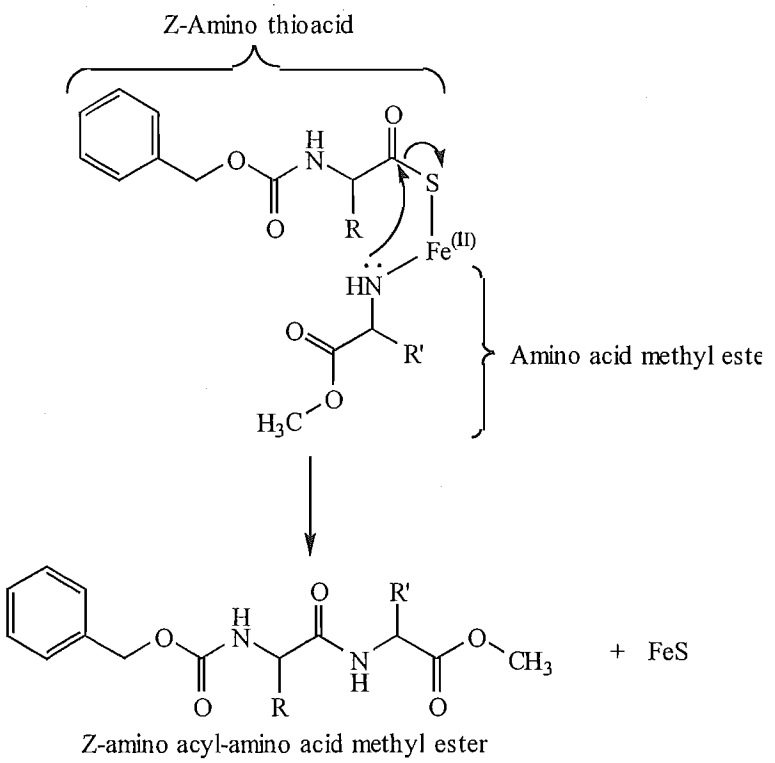

Figure 4.3: The hypothesised coordinative catalysis of amide bond formation by ferrous ions

However, both investigators reported the problem of oxidation of Fe(II) ions to Fe(III) ions in reaction mixtures, as was observed by the formation of insoluble ferric oxide, or "rust", over the course of an incubation. Catalysis could therefore not be conclusively attributed to ferrous ions alone.

The idea that ferric ion contaminants might be involved in the catalysis of amide bond formation was suggested by Meade, and drew support from a 1997 paper by Liu and Orgel[14], describing oxidative acylation by thioacids. Among other amide bond-forming reactions, acylation of phenylalanine by thioacetic acid was shown to occur at high rates in the presence of several oxidants, including ferricyanide salts. (Ferricyanide was used because it represents a soluble form of ferric ions that are incapable of coordinative chemistry with thioacids.) This result, along with the observation that ferrocyanide salts do not catalyse the reaction, led the investigators to postulate an oxidative mechanism for the acylation reaction. An oxidised thioacid disulfide was thought to be a reactive intermediate (figure 4.4), with the disulfide presenting a better leaving group than the thioacid. As in the Huber/Wächtershäuser, Meade, and Hampton experiments, competitive attack by water and amine nucleophiles on the thioacid species would give the acid or amide, respectively (figure 4.4).

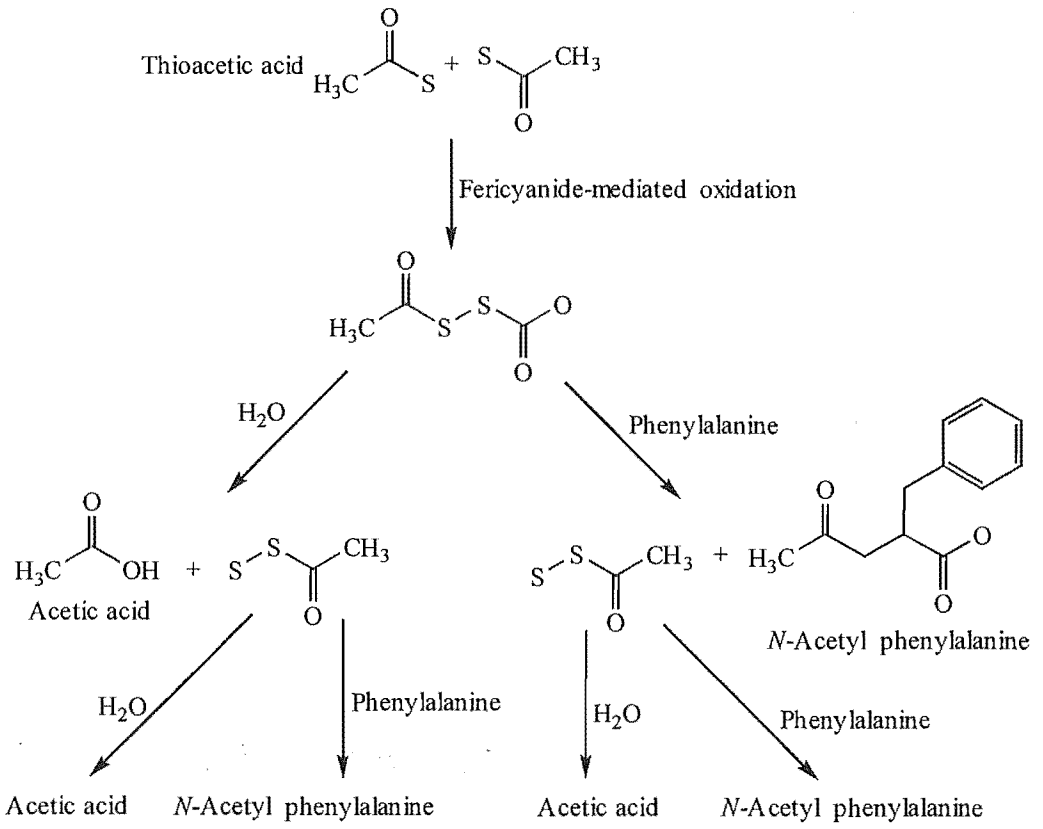

Figure 4.4: Oxidative acylation of phenylalanine by thioacetic acid

The remainder of section 4.2 investigates the respective contributions by ferrous and ferric ions in the reactions reported by Meade and Hampton concerning acylation of amines by thioacids.

4.2.1 The reaction system

In his experiments, which have been emulated in part of this work, Hampton investigated amide bond formation between Z-protected amino thioacids and amino acid methyl esters. Amino acid methyl esters were used as the nucleophilic species, so that nucleophilic attack would be directed towards the thioacid carbonyl carbon only. Z-protected amino thioacids were used because the Z protecting group eliminated nucleophilic participation by the thioacid amine, and provided a UV chromophore so that the progress of the reaction could be monitored by HPLC using a UV detector (figure 4.5).

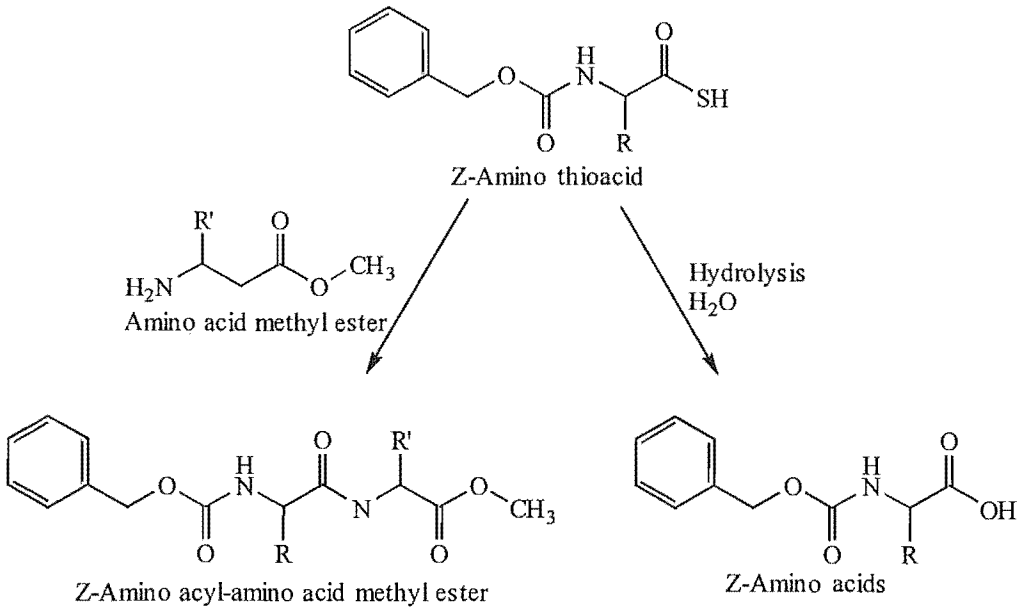

Figure 4.5: Species that will be detected by UV analysis in Hampton-type experiments

Hampton carried out experiments involving the incubation of solutions of Z-protected thioacids and amino acid methyl esters in the presence or absence of iron salts. Reaction mixtures were prepared for analysis by the addition of potassium cyanide, to coordinate iron and displace bound organic ligands. The resulting solutions were centrifuged and, after a variable amount of time, the supernatants were injected onto an HPLC for analysis by a UV detector. Using this methodology, he was able to demonstrate that the presence of iron caused significant increases in the rates of amide bond formation and hydrolysis; very little loss of thioacid was observed in trials run in the absence if iron. A pH of 6.8 (buffered with PIPES) and an incubation regime of 45°C for 24hr was found to be optimum. Specifically, when Z-alanine thioacid was incubated with alanine methyl ester (which is the combination of amino acid derivatives used in the following experiments) and iron ions under the specified reaction conditions, 44% of the thioacid reacted to give 17% acylation (to give the dipeptide) and 27% hydrolysis (to give Z-alanine).

To reiterate what was said above, iron was added to reaction mixtures as ferrous ions, but some oxidation to ferric ions (as observed by the formation of red/brown insoluble ferric oxide) occurred during the course of the reactions. It was therefore impossible to distinguish between a ferrous ion-mediated coordinative mechanism and a ferric ion-mediated oxidative mechanism for reaction of the thioacid. Therefore, in an attempt to assess the catalytic contribution by ferrous ions in Hampton's system, I repeated his experiments using more rigorously anaerobic reaction conditions.

4.2.2 Synthesis of reagents

The reaction system optimised by Hampton was initially chosen as a means of investigating ferrous ion-mediated catalysis because it had been well characterised, and involved HPLC analysis which gives accurate qualitative and quantitative data. It was first necessary to synthesise the amino acid derivatives to be used in the experiments. Amino acid methyl esters (figure 4.6) were synthesised by a protocol revised from that reported by Dondoni and Perrone[15]. Amino acids were stirred in methanol solutions with thionyl chloride; methyl ester synthesis therefore occurred through nucleophilic attack by methanol on the amino acid chlorides, which were generated *in situ* through reaction with thionyl chloride. The methyl esters were isolated and purified as their hydrochloride salts in yields of around 90%, and their structures confirmed using ^{1}H and ^{13}C NMR.

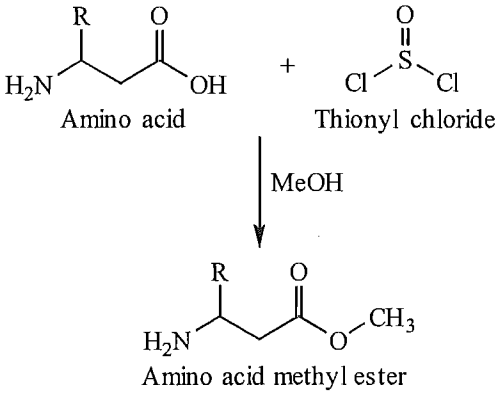

Figure 4.6: Synthesis of amino acid methyl esters

The synthesis of Z-amino thioacids was more involved, and involved several steps (fig 4.7). Z-protected amino acids were synthesised by the reaction of the respective amino acids with benzyl chloroformate (figure 4.7a), by the method of Bodanszky and Bodanszky[16]. The Z-protected amino acid products were then purified and reacted with *N*-hydroxy succinimide in the presence of dicyclohexyl carbodiimide to give the *N*-hydroxy succinimide esters (figure 4.7b), using a method reported by Anderson, Zimmerman and Callahan[17]. These esters were isolated, and using the method of Mitin and Zapevalova[18] were reacted with lithium sulfide to give the thioacids (figure 4.7c). These thioacids were purified and then dissolved in aqueous potassium hydroxide solutions to give a pH of 7, and the potassium salts of the thioacids (shown to be more stable over time[18]) isolated by freeze-drying these solutions, and purified by recrystallisation (figure 4.7d). ^{1}H and ^{13}C NMR was used to confirm the structure of the reaction products in each step.

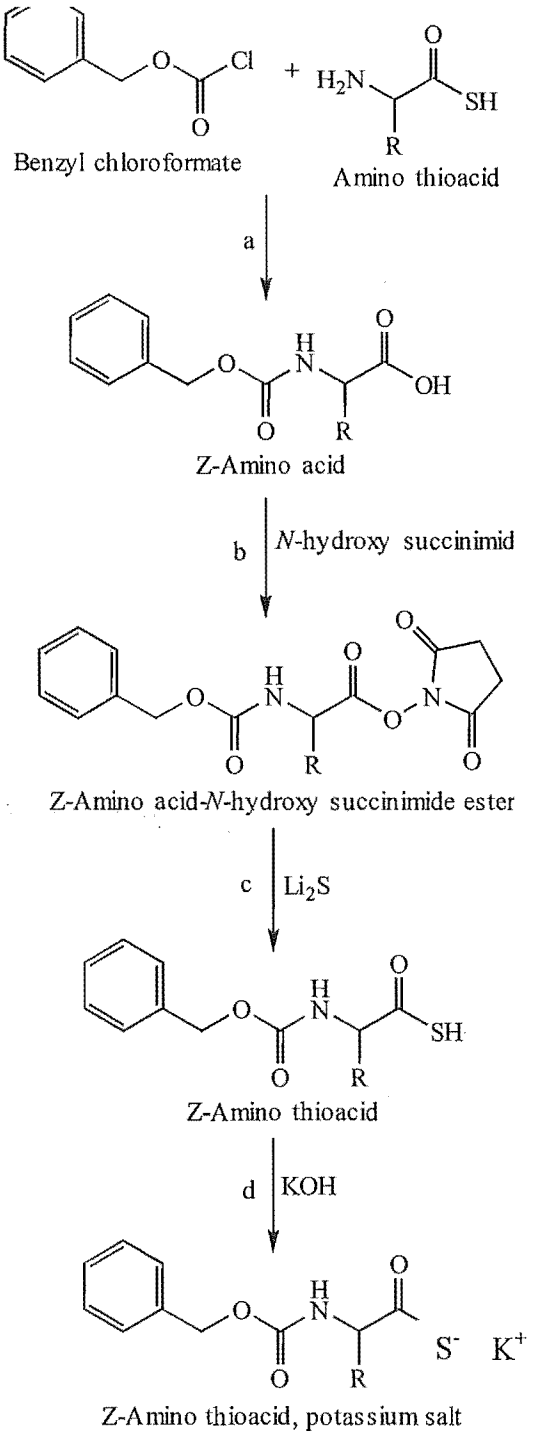

Figure4.7: Synthesis of potassium salts of Z-amino thioacids

Authentic samples of Z-amino acid-amino acid methyl ester dipeptide (figure 4.8) references were also synthesised by the method of Bodanszky and Bodanszky[19], for use in the identification of product peaks in HPLC traces. Z-protected amino acids were stirred with amino acid methyl esters in the presence of 1-hydroxy benzotriazole,

diisopropylethylamine and dicyclohexylcarbodiimide. The dipeptide products were purified by flash chromatography followed by recrystallisation, and their structures confirmed by ^{1}H and ^{13}C NMR.

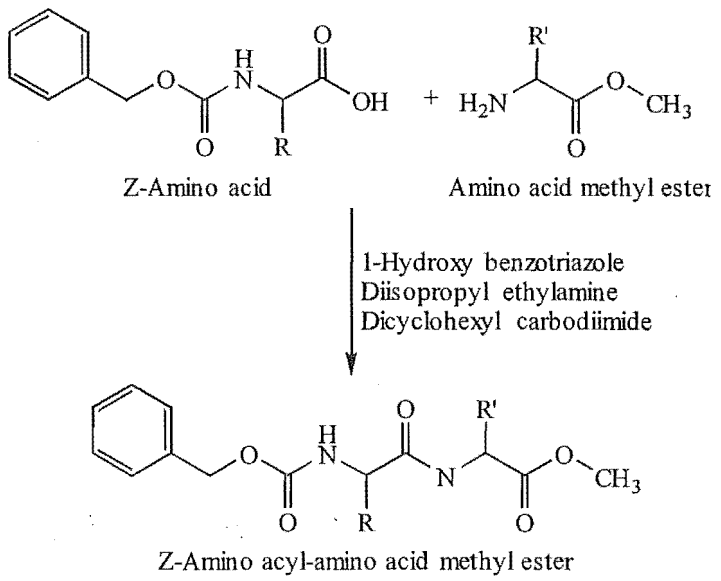

Figure 4.8: Synthesis of Z-amino acyl-amino acid methyl ester dipeptides

4.2.3 Minimising oxygen contaminants during sample incubation

Investigations into the effect of ferrous ions on the formation of amide bonds between amines and thioacids were to be carried out by incubating solutions of Z-alanine thioacid with Z-alanine methyl ester in the presence or absence of ferrous ions. With the reagents in hand, it was next necessary to minimise the amount of oxidation that could occur in trials designed to assess coordinative catalysis by ferrous ions. In Meade and Hampton's experiments the reaction solutions were initially rigorously degassed, but the reactions themselves were carried out in degassed Wheaton vials; the lids to these vials form a poor seal and allow the diffusion of oxygen, which would account for the observed formation of rust at the solvent-atmosphere interface. To overcome this problem, I used 0.5mL glass reaction vials fitted with rubber suba seals, which gave a much better seal than the Wheaton vial lids. In addition to this, reaction vials were incubated in argon-filled chambers, to further minimise diffusion of oxygen into reaction vials during incubation. No rust was seen in these trials, and

the results described in section 4.2.2 confirm that the incubation was now more rigorously anaerobic.

Another potential source of oxidation was during the workup period. In the past, potassium cyanide was added during sample workup to coordinate iron and displace bound organic ligands. The samples were then centrifuged and the iron cyanide-containing supernatants injected directly into the HPLC. The time lapse between cyanide addition and sample analysis varied from trial to trial, but may have been as long as two or three hours in some cases. This may have been long enough for a significant amount of ferrocyanide to oxidise to ferricyanide (vials were opened to the air during cyanide addition); this, in turn, could mediate oxidative acylation. (The results from section 1.2.7 show that ferricyanide will mediate amide bond formation with thioacids in a matter of minutes.) A new workup procedure was therefore developed. Addition of cyanide and centrifugation of the resulting solutions was carried out, as in the past. However, to minimise the amount of time that the thioacid spent in solution with iron cyanide complexes, the supernatants were passed through C_{18} cartridges immediately after centrifugation. The cartridges were washed with water, and the organics eluted with acetonitrile. These acetonitrile solutions were injected into the HPLC. This procedure ensured that the thioacid was isolated away from any Fe(III) ions within 10-20min of cyanide addition. A further, more practical, advantage of this new workup procedure was that problems involving precipitation of iron cyanide complexes within the HPLC could be avoided.

Two brief investigations were carried out to test the efficiency of this new workup protocol. The first involved testing the recovery of organics from reaction solutions when using the C_{18} cartridge. Z-L-alanine was chosen as a reference compound; it is relatively soluble in water and is one of the first peaks to be eluted during HPLC runs (see figure 4.9). It was quantitatively recovered using the C_{18} cartridge-workup procedure. This experiment was repeated using the Z-L-alanyl-alanine methyl ester dipeptide, which was also quantitatively recovered. This showed that minimal loss of organics was occurring when the C_{18} cartridge was washed with water, and that the acetonitrile was then able to elute effectively all of the bound organics from the cartridge.

The second experiment was designed to test any possible oxidative catalysis mediated by ferric ions arising *de novo* during this workup procedure (in the 10-20min time lapse between potassium cyanide addition and C_{18} cartridge-preparation). Control reactions that had not been incubated were analysed, i.e., reaction mixtures were analysed as soon as they had been generated. HPLC traces showed that virtually none of the thioacid had reacted, proving that ferricyanide-mediated oxidative acylation or hydrolysis reactions involving the thioacid during the 10-20min workup period were negligible.

With new incubation and workup protocols, it was hoped that the potential for oxidative acylation had been minimised. The results described above showed that oxidative catalysis during the workup was negligible, but to show that oxidative catalysis was not occurring during the incubation it was necessary to demonstrate that very little oxidation of Fe(II)→Fe(III) was occurring. The simplest way to do this was to assay for ferrous ion concentration before and after incubation.

4.2.4 Assaying for ferrous ions using phenanthroline

To determine the concentration of ferrous ions in reaction solutions, a colourimetric assay using phenanthroline was used[20]. Phenanthroline forms stable coloured complexes with Fe(II) and Fe(III) ions. It can be seen from the respective absorption profiles that at a wavelength of 512nm the Fe(II) complex absorbs strongly while the Fe(III) complex does not, and at 396nm both complexes absorb equally. It would therefore follow from this that concentration of Fe(II) and Fe(III) ions could be easily determined by measuring the absorptions of solutions at 512nm and 396nm. In practice, however, this was not so. It was found that total Fe^{n+} concentrations were not represented accurately enough by absorption readings taken at 396nm to allow the calculation of low concentrations of Fe(III) ions in the presence of high concentrations of Fe(II) ions; and therefore, absorption readings at 396nm were not used. In addition to this, it was found that within 1 minute of mixing, the absorption of a ferric ion/phenanthroline standard was negligible, but, over about 2 hours, this value climbed steadily before flattening off at the equivalent of about 40% of the absorption of an equimolar ferrous ion solution. The colour development in the ferrous ion/phenanthroline system, on the other hand, reached a maximum after 1

minute, and was stable for at least 20 minutes. Based on these observations, absorbency readings at 512nm were taken within 1 minute of mixing.

A calibration curve was constructed using standard solutions of ferrous sulphate (see figure 5. in section 5.4.9). There was a close correlation between Fe(II) ion concentration and absorbency at 512nm, and it was subsequently shown that Fe(II) ions could be accurately quantified in the presence of a 4-fold excess of Fe(III) ions.

The calibration curve was used to assay for the concentration of ferrous ions in reaction mixtures before and after incubation. Many experiments were run involving the incubation of solutions containing ferrous ions at a pH of 6.8 for 24hr at 45°C . Absorption readings of control solutions that had not been incubated (i.e., analysed immediately after the reaction mixtures were generated) gave values to within ±2% of the expected value, based on the mass of ferrous sulfate used in the trials. This level of experimental error could largely be explained by the error margins in measuring the small masses of ferrous sulfate being used. Analysis of solutions after incubation gave very similar results, showing that the amount of oxidation of ferrous ions in reaction solutions was below this experimental error of 2%.

4.2.5 Will ferrous ions catalyse the acylation reaction

Having minimised the potential for oxidative catalysis to occur during a typical reaction, it was now possible to investigate the ability of ferrous ions to catalyse the acylation of alanine methyl ester by Z-alanine thioacid. Solutions buffered to a pH of 6.8 and containing equimolar amounts of Z-D/L-alanine thioacid, L-alanine methyl ester and ferrous ions were incubated at 45°C for 24hr. Reactions without ferrous ions were also run in parallel. The solutions were worked up as specified above and analysed by HPLC. In stark contrast with the results reported by Hampton, there was no observable difference in peak sizes between trials that contained ferrous ions and those that didn't. Furthermore, the thioacid was not reacting to any appreciable extent.

This result showed that ferrous ions do not activate Z-alanine thioacid for nucleophilic attack under the specified reaction conditions. To investigate the effect of ferrous ions on thioacid chemistry under conditions in which the thioacid would react, the

experiment was repeated with an increased incubation temperature and time of 65°C for 3 days. A black iron-sulfur precipitate formed over the course of the incubation in reaction solutions that contained ferrous ions, which can be taken as an indicator of liberation of sulfide ions through nucleophilic attack upon the thioacid. The solutions were worked up and analysed by HPLC (see figure 4.9).

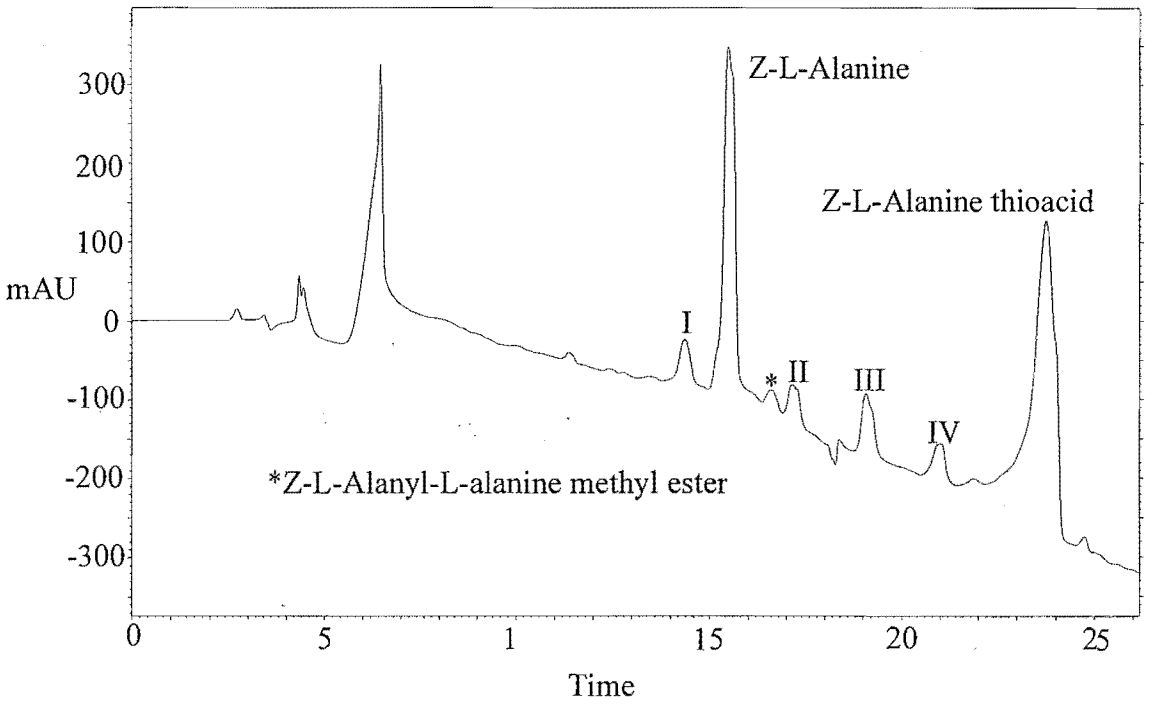

Figure 4.9: An HPLC trace from a reaction mixture containing ferrous ions, Z-alanine and L-alanine methyl ester after 3 days at 65°C

Under these new incubation conditions around half of the thioacid reacted. Seven peaks can be seen in the HPLC trace; three of those correspond to Z-alanine (the product of the hydrolysis reaction), the Z-alanyl-alanine methyl ester dipeptide (the product of the acylation reaction), and unreacted Z-alanine thioacid. Comparison of the peak areas in HPLC traces of experiments run in the presence or absence of ferrous ions showed that ferrous ions did not cause a significant increase in the rate of acylation, while the rate of hydrolysis was seen to increase by a factor of approximately 1.2. A general type of thioacid activation, involving ferrous ions drawing electron density away from the carbonyl carbon of bound thioacids, is

therefore probably not responsible for the ferrous ion-dependent increase in the rate of hydrolysis because an increase in nucleophilic attack by the amine would also be expected. Rather, a coordinative mechanism for ferrous ion-mediated catalysis of hydrolysis, involving metal-bound water molecules, was most likely occurring.

The appearance and size of the four unidentified peaks observed in the HPLC analyses was not ferrous ion-dependent. To test whether they corresponded to possible breakdown-products of the dipeptide (which is formed during the reaction), a reference sample of the dipeptide was incubated under the new and harsher conditions. A near-saturated solution of the relatively insoluble Z-alanyl-alanine methyl ester dipeptide was incubated with ferrous ions at a pH of 6.8 for 3 days at 65°C. Three new peaks were observed in the HPLC analysis, which correspond to three of the unidentified peaks observed previously (figure 4.9, peaks I, II and III). The origin of one of the unknown compounds giving rise to a peak in the HPLC analyses of reaction mixtures from amide bond-forming experiments (fig4.9, peak IV) remains unknown.

Taken together, these results strongly suggest that catalysis of both the acylation and the hydrolysis reaction described by Hampton were primarily due to an oxidative mechanism. Z-L-alanine thioacid did not react under the conditions that had been reported for ferrous ion-mediated catalysis, and when incubation conditions were used that forced the thioacid to react no ferrous ion-mediated catalysis of acylation of the amine was detected.

Meade was unable to demonstrate amide bond formation between amino thioacids and amino acids, but she did detect amide bond formation in analogous trials involving thioacetic acid. One rationale for this result is that the greater steric bulk of amino thioacids, when compared with thioacetic acid, might inhibit ferrous ion-mediated coordinative catalysis of amide bond formation. The next section investigates this possibility.

4.2.6 Does the steric bulk of amino thioacids inhibit coordinative catalysis?

To test the possibility that steric bulk might inhibit coordinative catalysis of amide bond formation, acylation reactions between thioacetic acid and phenylalanine were

carried out in the presence and absence of ferrous ions. Phenylalanine was chosen as the amino acid nucleophile because the benzene chromophore allowed the progress of the reaction to be monitored by HPLC with a UV detector (figure).

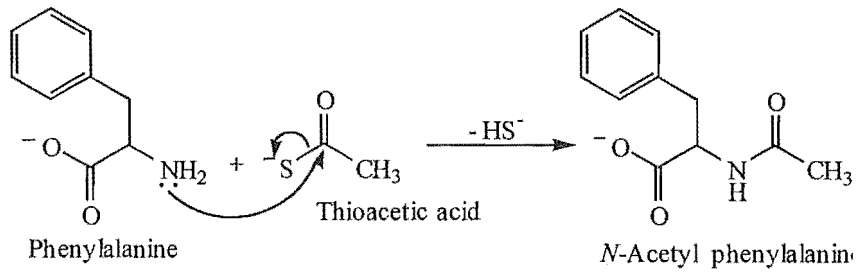

Figure 4.10: A chromophore-containing system designed to investigate acylation of amines by thioacetic acid

Equimolar solutions of phenylalanine, thioacetic acid and ferrous ions, buffered to a pH of 6.8, were incubated at 50°C for 24hr. (An incubation of 65°C for 3 days, as was used in the experiments described in section 4.2.4, was inappropriate due to the higher reactivity of thioacetic acid compared with Z-alanine thioacid.) A control experiment without ferrous ions was also run in parallel. Comparison of the HPLC traces of the two reactions showed them to be near identical, with both showing 21% acylation of phenylalanine, which was surprisingly high. None the less, ferrous ion-specific effects in the acylation of phenylalanine by thioacetic acid were not demonstrated.

Again, these results strongly suggest that a ferric ion-mediated oxidative mechanism of amide bond formation was occurring in the experiments reported by Meade and Hampton. Section 1.2.7 describes experiments designed to test the effect of oxidation on the Hampton- and Meade-type systems.

4.2.7 Confirming oxidative acylation

Having established that ferrous ions do not catalyse the acylation of amino acids by thioacids (at least under the conditions investigated), it was necessary to evaluate the phenomenon of oxidative acylation by thioacids as a possible explanation for Meade and Hampton's results. Several experiments were run to investigate this. The logical

first step was to directly replicate one of the 1997 Liu and Orgel experiments, to confirm the ferricyanide-mediated acylation of phenylalanine by thioacetic acid. Solutions buffered to a pH of 6.35 and containing potassium ferricyanide, thioacetic acid, and L-phenylalanine were stirred at room temperature. Aliquots were taken after 20min and 15hrs, worked up using the C_{18} cartridge methodology, and analysed by HPLC. Three peaks appeared; two of which were shown (by spiking with authentic samples) to be phenylalanine and *N*-acetyl phenylalanine. A summary of all the results are given in table 4.1.

Table 4.1: A summary of results for ferricyanide-mediated acylation reactions involving phenylalanine and thioacetic acid

phenylalanine concentration	Incubation time	%Phenylalanine*	%*N*-acetyl phenylalanine*	% Unknown compound*
25mM	20min	19	18	63
25mM	15hr	20	77	3
75mM	20min	34	29	37
75mM	15hr	36	63	1

*values are given as % of the total composition of chromophore-containing molecules, as determined from the HPLC trace.

The identities of the phenylalanine and the *N*-acetyl phenylalanine peaks were confirmed using HPLC by spiking reaction solutions with authentic samples of reference compounds. Experiments incubated in the absence of ferricyanide gave very little acylation, confirming that ferricyanide-mediated catalysis was occurring.

It can be seen that after 20min of reaction time a third peak appears in HPLC traces, and that the molecule corresponding to this peak is present at relatively high concentrations at this time. (It should be mentioned here that inherent in the relative ratio values given in table 4.1 is the assumption that the unknown compound contained only one benzene ring chromophore.) The unidentified compound that

gave rise to this third peak is all but lost from reaction mixtures after 15hr. The compound is unidentified and presumed to be a reaction intermediate.

In order to characterise the reaction more fully, samples were subject to analysis by HPLC/MS. On-line analysis of eluant by negative-ion MS using atmospheric pressure chemical ionisation confirmed the identity of phenylalanine and *N*-acetyl phenylalanine, but no ions were observed corresponding to the unidentified compound described above. The identity of this compound therefore remains unknown.

To confirm that oxidative acylation occurs in analogous reactions involving amino acid methyl ester nucleophiles and Z-protected amino thioacids, solutions of alanine methyl ester and Z-alanine thioacid were incubated at room temperature for 20hrs in the presence of a two-fold excess of potassium ferricyanide at a pH of 6.8. (The choice of reagents and stoichiometries was based on the trials attempting to replicate Hampton's results, described in section 4.2.5.) Analysis of this solution by HPLC showed 2 peaks which were identified as Z-alanine and the Z-alanyl-alanine methyl ester dipeptide, in a ratio of roughly 1:2, respectively. Ferricyanide-mediated acylation of the amine had occurred efficiently, supporting the idea that the acylation observed in the Hampton experiments was probably due to an oxidative process.

The ability of ferric ions, added in the form of ferric chloride, to catalyse oxidative acylation was next investigated. Solutions buffered to a pH of 6.8 and containing Z-alanine thioacid, alanine methyl ester and an excess of ferric chloride were incubated for three days at 65°C. Analysis by HPLC showed three peaks; two of these were identified as Z-alanine and the Z-L-alanyl-alanine methyl ester dipeptide (figure 4.11). The identity of the compound that gave rise to the third peak is unknown.

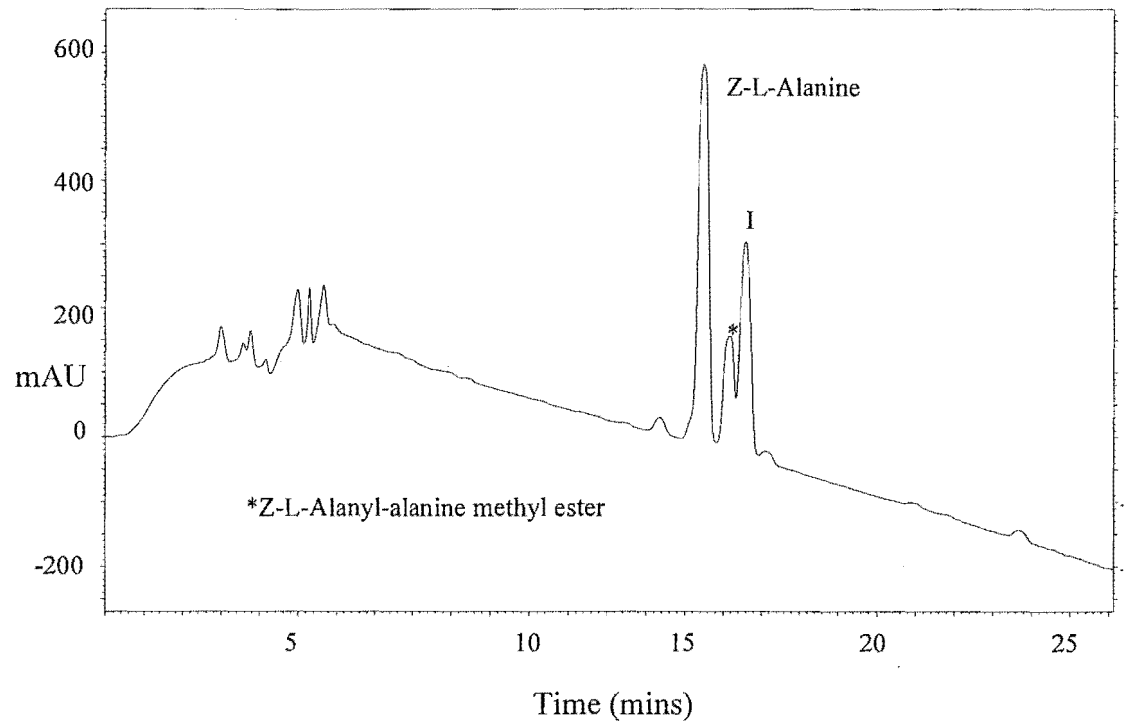

Figure 4.11: HPLC trace showing catalysis of amide bond formation by Fe(II) ions

The deliberate inclusion of ferric chloride in reaction mixtures therefore increased the rates of both hydrolysis and acylation. This catalysis is most likely to have occurred by an oxidative mechanism. These results lead to the conclusion that the acylation chemistry in the Meade and Hampton experiments was primarily due to ferric ions generated *in situ* during the course of the reactions.

4.3 Catalysis of amide bond formation by nickel ions

The reaction system reported by Huber and Wächterschäuser in their 1997 paper[10] contained a strongly reducing mixture of Fe(II) ions, Ni(II) ions and sulfide ions. It was possible, however, that some oxidised metal ion species may have been present, especially given that the reduction of organic species was demonstrated. The previous section reported an examination into possible catalysis of thioacid chemistry by

ferrous ions and ferric ions, and this section reports a similar investigation into catalysis by Ni(II) ions in the presence or absence of molecular oxygen as an oxidant.

Solutions buffered to a pH of 6.8 and containing equimolar amounts of Ni(II) ions, alanine methyl ester and Z-alanine thioacid were incubated for 3 days at 65°C. Trials without Ni(II) ions were also run in parallel. The stoichiometries and reaction conditions were identical to those used in the ferrous ion trials. Nickel sulfide, a more shiny-looking precipitate than iron sulfide, formed over the course of the reaction; an indication of the liberation of free sulfide by nucleophilic attack on the thioacid. Solutions were worked up exactly as in the Fe(II) ion trials, because cyanide ions will also form stable complexes with Ni(II) ions. Analysis by HPLC gave very similar results as for the ferrous ion reactions (see section 4.6), and showed that the presence of Ni(II) ions increased the level of hydrolysis by a factor of about 1.4, while the level of acylation increased by a factor of approximately 2. This result is discussed in section 1.4, in light of results presented there.

Interestingly, when these experiments were repeated without degassing the reaction solutions, significant increases in the extents of acylation were sometimes detected. This result was not quantitatively reproducible, with the extent of acylation varying considerably between experiments. This increase in acylation (when compared with experiments involving degassed reaction solutions) is presumably as a direct result of the presence of oxygen, and possible explanations for this are discussed in section 1.4, in light of the results presented there.

4.4 Catalysis of amide bond formation by nickel and iron sulfides

The reaction system reported by Huber and Wächterschäuser[10] contained a (Ni/Fe)S coprecipitate. Investigations were therefore carried out to investigate the capacities of NiS, FeS and (Fe/Ni)S precipitates to catalyse the acylation reaction. Solutions at a pH of 6.8 containing equimolar amounts of Z-alanine thioacid, alanine methyl ester, and an FeS precipitate, NiS precipitate, or (Fe/Ni)S coprecipitate were incubated at 65°C for 3 days. The stoichiometries of the reagents (including the metal ions) were the same as in the free Ni(II) ion and Fe(II) ion experiments. Solutions were worked

up as in previous trials (addition of cyanide ions, centrifugation and purification of organics using C_{18} cartridges) and analysed by HPLC. The results from these analyses are summarised in table.

Type of precipitate	% Thioacid loss*	% Hydrolysis*	% Acylation*
None	30	22	8
FeS	59	44	15
NiS	70	33	37
FeS/NiS	62	41	21

*values are given as a percentage of the thioacid present at the start of the reaction

Table 4.2: Catalysis of Z-alanine thioacid reaction by NiS, FeS and NiS/FeS

It can be seen that ferrous sulfide doubles the loss of the thioacid, and the rates of both hydrolysis and acylation are increased by a factor of two. Given that free Fe(II) ions in solution catalyse only the hydrolysis reaction, it can be concluded that ferrous sulfide is a better catalyst of the acylation chemistry than ferrous ions alone. This is most likely due to changes in the coordinative chemistry of ferrous ion centres relative to each other when they are bound within an FeS lattice.

Nickel sulfide increases the rate of hydrolysis to a similar extent as free Ni(II) ions in solution. Amide bond formation, however, is increased by a factor of 4.5 in the presence of NiS, which is significantly more reaction than is seen in the analogous anaerobic reactions containing dissolved Ni(II) ions. This increase in acylation is therefore presumably due to coordinative chemistry involving adjacent Ni(II) ion centres on the precipitate surface (figure 4.12).

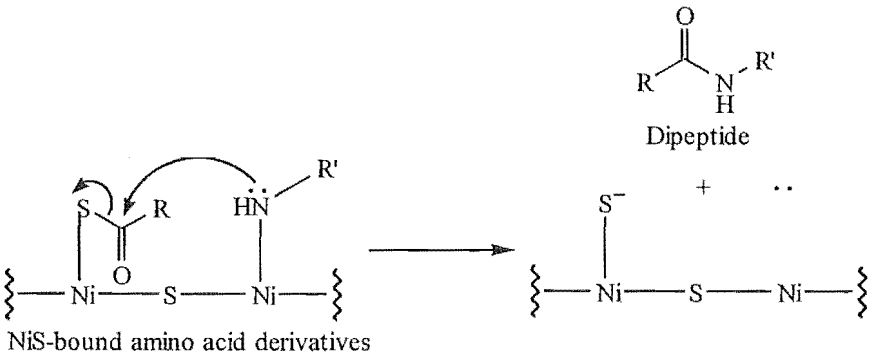

Figure 4.12: Catalysis of amide bond formation by NiS precipitates

Finally, it can be seen from table that ferrous sulfide appears to "poison" the nickel sulfide catalyst (with respect to the acylation chemistry), with the levels of hydrolysis and amide bond-formation catalysed by FeS/NiS co-precipitates falling between those for FeS and NiS alone.

The catalysis of amide bond formation by NiS precipitates may help to explain the increased rates of amide bond formation observed in free Ni(II) ion trials (results were presented in section 1.3). Precipitation of nickel sulfide occurred during the course of reactions involving free Ni(II) ions in solutions, and it was probably this NiS precipitate that catalysed the formation of amide bonds.

Catalysis by nickel sulfide may also help to explain why an increase in the rate of acylation was seen in experiments carried out in the presence of nickel ions without degassing reaction solutions, compared to equivalent experiments where the reaction solutions were degassed (results are presented in section 1.3). As was mentioned in the previous section, the presence of oxygen is presumably the reason for the increase in acylation, implicating an oxidative mechanism in this process. Coordinative catalysis by thioacid-bound Ni(III) ions is an unlikely possibility, as any thioacid-bound Ni(III) ions would be expected to be reduced to Ni(II) ions with the concomitant oxidation of the thioacid to the corresponding disulfide species; it is this disulfide species that was probably involved in the increase in acylation. The increase in acylation cannot be attributed to nucleophilic attack on the disulfide species alone, however, as nucleophilic attack on the disulfide by the amino acid methyl ester would

compete with attack by water, and a significant increase in the extent of hydrolysis was not detected. The most likely mechanism that explains the increase in amide bond formation involves nucleophilic attack (by both water and the amine nucleophiles) on the disulfide species, with the disulfide ions liberated precipitating out of solution with nickel ions. These nickel disulfide precipitates could then have catalysed the acylation chemistry. Further support for this model comes from analogous experiments run in the presence of iron ions (as opposed to nickel ions). The presence of oxygen in these experiments did not cause an increase in acylation, reflecting the observation that iron-sulfur precipitates were not shown to catalyse the acylation reaction.

4.5 Conclusions

This chapter was concerned with investigating the possibility that an (Fe/Ni)S precipitate might catalyse the acylation of amines by thioacids, as suggested by Huber and Wächtershäuser[10]. Research into this possibility had already been carried out, with both Meade[12] and Hampton[13] reporting an increase in amide bond formation between amines and thioacids in the presence of iron ions. A ferrous ion-mediated co-ordinative mechanism had been proposed for this process, but a ferric ion-mediated oxidative mechanism was also possible. Results presented in section 4.2 of this chapter show that ferrous ions do not cause an increase in the rate of acylation of amines by thioacids, whereas ferric ions do. The coordination chemistry of ferric ions is not implicated in this process, and an oxidative mechanism is presumed to be responsible for the increase in reaction rate. These results strongly suggest that the increase in acylation of amines by thioacids in the presence of iron ions reported by Meade and Hampton was mediated by ferric ions forming *in situ* by the oxidation of ferrous ions over the course of the reactions.

It was next discovered that nickel sulfide precipitates catalyse the acylation of amines by thioacids by a factor of 4.5, and that the rate of this increase in acylation is much larger than the increase in the rate of hydrolysis of the thioacid, which increased by a factor of 1.5 in the presence of NiS. This minimal loss of thioacid through hydrolysis

is important when considering the potential primordial occurrence of this type of chemistry, given that the prebiotic supply of thioacids may have been very limited.

Free Ni(II) ions in solution did not catalyse the acylation chemistry to the same extent as NiS, suggesting that this type of chemistry is more relevant to environments rich in sulfide. Deep-sea hydrothermal vent systems are the most obvious example of sulfur-rich prebiotic environments, and NiS present in these areas[21].

Ferrous sulfide catalyses amide bond formation between amines and thioacids to a greater degree than free ferrous ions in solution, but is a poor catalyst compared to nickel sulfide; in fact, ferrous sulfide "poisons" the nickel sulfide catalyst. These results suggest that the nickel sulfide component, rather than the iron sulfide component, in the FeS/NiS coprecipitate used in the Huber-Wächtershäuser experiments was probably the active catalyst in the amide bond-forming reaction.

The efficient catalysis of amide bond formation between thioacids and amines by nickel sulfide precipitates under anaerobic conditions is an interesting phenomenon, deserving further investigation. Nickel sulfide precipitates would have been present on prebiotic earth[21], and thioacids and amines are potential prebiotic reagents; the formation of amide bonds over NiS precipitates may therefore have been an important reaction involved in the origins of life.

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Chapter 5 Experimental

5.1 General experimental

Nuclear magnetic resonance (NMR) spectroscopy was performed on a Varian XL 300 instrument. This spectrometer operates at 300MHz for ^{1}H nuclei, 75MHz for ^{13}C nuclei, and 120MHz for ^{31}P nuclei. The probe temperature was 23°C. Chemical shifts in this thesis are represented in parts per million on the δ scale; ^{1}H and ^{13}C peaks are referenced to residual solvent peaks in d_6-DMSO and D_2O. A sample of 85% phosphoric acid in deuterium oxide was used to calibrate the spectrometer for ^{31}P NMR. Multiplicities are denoted as singlet (s), broad singlet (bs), doublet (d), triplet (t), quartet (q), octet (o) or multiplet (m).

Reverse phase HPLC analyses with UV visualisation were carried out on either a Shimadzu LC-10AD VP liquid chromatograph, with an SPD M10A VP photodiode array detector; or a Phillips PU4120 Diode Array Detector interfaced to a personal computer running Phillips PU6003 Diode Array Detector System Software (V3.0) and a colour plotter. A Phenomenex Prodigy C_{18} 5-ODS (3μ, 250 x 4.6mm) column was used in both machines. Samples were run with variable amounts of water and acetonitrile. Water for HPLC analysis was produced with a Milli-Q Water Purification System(Millipore) and contained 0.05% TFA, and the acetonitrile used was HPLC grade. A flow rate of $1mLmin^{-1}$ was used in all analyses, and absorbencies at 203nm were used to quantify compounds.

HPLC/MS analyses were carried out using a Micromass LCT mass spectromoter with atmospheric pressure chemical ionisation (APCI). A Waters 2790 liquid chromatograph was used to separate reaction mixture components and was connected to a 996 PDA detector. Samples were run with variable amounts of HPLC-grade acetonitrile and milli-Q water in the presence of 0.1% formic acid. A flow rate of $0.2mLmin^{-1}$ was used.

Analytical thin layer chromotography was conducted on aluminium-backed Merck Kieselgel KG60F_{254} silica plates. When appropriate, visualisation was conducted by short wavelength UV light.

Flash chromatography was conducted on Merck silica 60.

Infra-red data was obtained on a Shimadzu 8201PC series FTIR interfaced with an Intel-486PC running Shimadzu's Hyper IR software.

High speed centrifugation was performed on an Eppendorf Centrifuge 5403, on a small scale (<1.5mL) at 15000 r.p.m., and on a large at up to 5000 r.p.m.

Melting points (m.p.) were recorded on an Electrochemical melting point apparatus and are uncorrected.

All pH measurements on volumes less than 5mL were performed using universal indicator paper (pH 0-14) to obtain the approximate value and then pH with smaller increments (eg. pH 1-3). pH measurements for volumes larger than 5mL were performed using a Jenway 3020 pH meter fitted with a Schott Gerate N37BNC electrode, calibrated against standard buffers at pH 4, 7 and 9.

Unless otherwise stated, all materials were obtained from Sigma Chemical Company Ltd., Aldrich Chemicals or BDH Laboratory Supplies, and were generally of analytical grade.

Solvents and reagents were purified according to well known procedures[1].

5.2 General anaerobic manipulations

All reactions involving ferrous ions were carried out in the absence of oxygen, under an atmosphere of argon.

5.2.1 The degassing apparatus

The degassing apparatus was comprised of a cylinder supplying a positive pressure of oxygen-free argon, a high vacuum pump, and two-way taps to allow switching between the two. Surplus argon gas was bubbled through a small vial of silicone oil, which gave the closed argon system a slight positive pressure, and prevented the back-diffusion of air. Glass vials to be degassed were fitted with airtight rubber suba seals, which provided septa that could be punctured by syringe needles, without compromising the airtight seals. These vials were placed on the degassing apparatus via needle-fitted hoses leading from the two-way taps. Degassing of a flask and its contents typically involved four cycles of applying a high vacuum for 3-5 minutes (more time was needed to remove oxygen from water and buffer solutions) followed by flushing with argon.

5.2.2 Anaerobic transfer of liquids

Liquids were transferred between vials using plastic syringes fitted with needles. Typically, a needle was inserted through the suba seal septum of a degassed liquid-containing flask, still attached to the degassing apparatus, and three brief degassing cycles were carried out, to empty the syringe of oxygen. With a positive pressure of argon, the liquid was then drawn into the syringe and quickly transferred to the appropriate flask. The resulting solution was immediately degassed with two brief vacuum/argon cycles, to minimise any oxygen contamination that could have occurred during the procedure.

5.2.3 Generation of reaction mixtures

Solutions of reagents were generated in the following manner. Reagents were weighed into vials, which were then fitted with suba seals and degassed. Water or buffer solutions were degassed, and an appropriate volume transferred to the degassed reagent-containing vials. Magnetic fleas were added to reaction vials, which were then fitted with suba seals and degassed. Appropriate aliquots of the degassed reagent solutions were transferred to reaction vials.

5.2.4 Incubation

Degassed reaction vials were incubated in argon-filled chambers, to further minimise the possibility of oxygen contamination. Reaction vials were placed in a chamber,

which in turn was connected to the degassing apparatus by a rubber hose and degassed with three vacuum/argon cycles. The chambers were then sealed via a tap. The chambers were heated by an oil bath on a heating mantle with magnetic stirrer. The chambers contained a fitting that allowed a thermometer to be inserted into the local environment of the reaction vessels. This compromised the air-tight seal, however, so temperature readings were only taken immediately prior to and following incubation periods. The two temperatures never varied by more than 2°C, regardless of the respective times of the day that the readings were taken. Incubation times and temperatures varied between trials, as specified in the text.

5.3 Sample workup using a C_{18} cartridge

HPLC-grade acetonitrile and milli Q water were used. The Bakerbond C_{18} cartridge was first prewashed with 2mL acetonitrile, followed by 1.5mL 50% acetonitrile and then 1.5mL water. An aqueous reaction solution was then passed through the column, which was washed with 1mL water. The washings were discarded, and a fresh collection vessel fitted onto the apparatus. The column-bound organics were eluted with 2mL of acetonitrile, which was collected and filtered, to give a solution ready for analysis by HPLC. The column was washed between samples with acetonitrile, water/acetonitrile and water solutions, as above.

5.4 Experimental from chapter 2

5.4.1 Purification of cysteamine

Cysteamine (2.0g) was weighed into a 10mL round-bottomed flask and a flea was included. A suba-seal was fitted and the vial and its contents degassed and filled with argon. The flask was heated in an oil bath and a steady flow of argon was passed through the flask as 5mL of degassed ethanol was added. The solution was stirred until the ethanol began to boil, by which time the cysteamine had dissolved. The solution was allowed to cool under a positive pressure of argon, during which time crystals of cysteamine formed. Any cystamine (the oxidised cysteamine disulfide derivative) remained in the ethanol. The crystals were filtered and washed with

degassed ethanol (2x3mL) before being placed under vacuum to remove residual ethanol. Pure cysteamine was obtained in a yield of 60% and stored under argon at 4°C.

5.4.2 Synthesis of *N,S*-diacetylcysteamine[2]

1.13g of cysteamine (14.7mmol) was weighed into a 100mL 3-necked round-bottomed flask. A magnetic flea was included, and the flask placed in an ice bath over a magnetic stirrer. A pH meter was fitted into one neck and a dropping funnel containing acetic anhydride (4.2mL, 44.0mmol, 3 mol. equiv.) into another. 20mL of water were added and the cysteamine was dissolved with stirring. 2M hydrochloric acid was added to bring the pH down to 8. The acetic anhydride was added drop-wise over twenty minutes, with simultaneous addition of 8M potassium hydroxide to keep the pH around 8. At the end of the addition the pH was adjusted to 7 with the hydrochloric acid solution and left to stir at room temperature for thirty minutes. The solution was then saturated with sodium chloride and extracted with dichloromethane (5x20mL). The dichloromethane extractions were pooled, dried with magnesium sulfate and filtered. The resulting solution was placed on a rotary evaporator, and the dichloromethane was removed to leave a colourless oil. This oil was placed under a high vacuum to remove any remaining solvent. NMR showed the product to be free of contaminants.

Yield: 2.31g (98%)

^{1}H NMR ($CDCl_3$): δ 1.93 (s, 3H, SCOC**H**$_3$), 2.32 (s, 3H, NHCOC**H**$_3$), 2.98 (t, 2H, SC**H**$_2$), 3.39 (q, 2H, (NHC**H**$_2$), 6.10 (bs, 1H, N**H**)

^{13}C NMR ($CDCl_3$): δ 21.92 (SCO**C**H$_3$), 28.09 (S**C**H$_2$), 29.84 (NHCO**C**H$_3$), 38.53 (NH**C**H$_2$), 174.02 (**C**O), 200.08 (**C**O)

5.4.3 Synthesis of hydroxyapatite[3]

Solutions of calcium nitrate (75mL, 0.133M), diammonium hydrogen phosphate (75mL, 0.072M), and ammonium acetate (270mL, 1M) were generated using degassed water. Concentrated, carbon dioxide-free aqueous ammonia solution was

used to adjust the pH of the solutions to 9-10. With vigorous stirring, the calcium nitrate and ammonium phosphate solutions were slowly added to the ammonium acetate solution in a 500mL round-bottomed flask. A fine white precipitate formed immediately. The ammonia solution was added intermittently to maintain the pH between 9 and 10. A reflux condenser was fitted to the round-bottomed flask, which was then placed in a heated oil bath. The solution was gently boiled for three hours, and then allowed to cool to room temperature. The fine white precipitate of hydroxyapatite was isolated by centrifugation and washed with water (3x160mL) followed by 95% ethanol (1x160mL). The precipitate was transferred to a round-bottomed flask and left overnight in a vacuum desiccator over phosphorous pentoxide.

Yield: 0.80g, 89%

5.4.4 Experimental from section 2.2.5

Two reaction mixtures were generated anaerobically. The first ("reaction 1") contained ferrous ions (60mM), sulfide ions (50mM), phosphate ions (400mM), *N,S*-diacetyl cysteamine (25mM) and pyrophosphate ions (3mM); and the second ("reaction 2)contained ferrous ions (60mM), sulfide ions (50mM), phosphate ions (400mM), thioacetic acid (25mM) and pyrophosphate ions (0.5MmM). These solutions were incubated in argon-filled chambers at 55°C. Aliquots were taken at specified times and mixed with 100μL aliquots of a solution of potassium cyanide (3M) in D_2O. The resulting solutions were shaken and left to stand for 10-15min, then centrifuged at 15000rpm for 5min. The supernatants were analysed by ^{1}H and ^{31}P NMR. NMR results are shown below in table 5.1

Table 5.1: NMR data from the experiments described in section 2.2.5

Reaction mixture	Time (days)	% hydrolysis	% pyrophosphate signal*
"Reaction 1"	2	15	8.4
	4	24	7.5
	7	33	6.3
	10	43	3.9
"Reaction 2"	2	13	1.4
	4	16	1.5
	7	18	0.9
	10	26	1.1

*these values were obtained by a direct comparison of pyrophosphate and phosphate peak areas in the ^{31}P NMR results

5.4.5 Experimental from section 2.3

A solution of acetyl phosphate (50mM) and potassium dihydrogen phosphate (180mM) together in 2.5mL 1M MES (pH 6.5) was mixed with a solution of ferrous sulfate (200mM) in 2.5mL water anaerobic conditions, in a degassed reaction vial. A white precipitate formed immediately. The mixture was incubated with stirring under an atmosphere of argon for 24hrs at 40°C. Potassium cyanide solution (1mL, 3M) was added, the resulting solution shaken vigorously, left to stand for 10min then centrifuged at 15000rpm for 5min. 0.7mL of the supernatant was added to 0.1mL of D_2O and analysed by ^{31}P NMR. A pyrophosphate signal was seen at δ -5.5ppm (the identity of which was confirmed by spiking the solution with potassium pyrophosphate), and the ratio of the phosphate:pyrophosphate peak areas was 93.5:6.5.

5.4.6 Experimental from section 2.4.1

A general experimental protocol is outlined below, with specific reaction details being given in tabular form (see table 5.2 for reagent stoichiometries, order of addition of reagents during generation of reaction mixtures, and temperature and duration of

incubations). Reactions are numbered in chronological order from 1-18 for ease of reference.

Solutions of ferrous sulfate in water, sodium hydrogen sulfide in water, potassium dihydrogen phosphate in 1M MES (pH 6.5) and *N,S*-diacetyl cysteamine in 1M MES (pH 6.5) were generated anaerobically. Generally, the stoichiometries of these reagent solutions allowed 0.25mL of each to be transferred to reaction vials to give 1mL reaction mixtures at the desired stoichiometry. Occassionally larger volumes were used to allow several aliquots to be taken over the course of the reactions. When solutions of ferrous ions were mixed with solutions of either sulfide ions or phosphate ions black FeS precipitates and white $FePO_4$ precipitates, respectively, formed immediately. In control experiments water was added in place of the ferrous ion or sulfide ion solutions. All reaction mixtures were incubated in argon filled chambers. At the end of an incubation, a 12-fold molar excess (relative to ferrous ion concentration) of potassium cyanide was added as a 3M aqueous solution. The solutions were shaken and left to stand for 10-15min, then centrifuged at 15000rpm for 5min. 0.7mL of the supernatants were mixed with 0.1mL aliquots of D_2O and analysed by ^1H and ^{31}P NMR. ^1H NMR was used to assay for loss of thioester or thioacid; the large water peak was calibrated to δ 4.60ppm, and the *N,S*-diacetyl cysteamine, *N*-acetyl cysteamine and acetic acid gave clear signals at δ_H 1.87, 1.85 and 1.83, respectively. ^{31}P NMR was used to assay for pyrophosphate production. The large phosphate signal was calibrated to δ 3.37ppm, and pyrophosphate signals appeared around δ –5.5ppm (the chemical shifts varied with pH). When necessary, samples were spiked with pyrophosphate either directly, or by the inclusion of an NMR tube-insert containing a pyrophosphate solution during analysis.

No pyrophosphate production was detected in any of the reaction solutions.

Table 5.2: A summary of experiments involving attempted pyrophosphate production with *N,S*-diacetyl cysteamine as condensing reagent

		Reagent concentrations (mM)				Incubation regime		
# and notes	volume (mL)	Thioester	Ferrous ions	Sulfide ions	Phosphate ions	Time (days)	Temperature (°C)	Hydrolysis (%)
1[a]	5	50	100	-	50	3	40	-
2[a]	8	600	60	60	55	7	40	50
3[a]	8	600	60	60	55	12	40	-
4[a]	5	600	60	-	55	7	40	65
5[a]	5	600	60	-	55	12	40	-
6[a]	5	600	60	-	55	12	63	-
7[a]	5	600	60	-	55	12	85	-
8[b]	6	400	85	100	60	10	55	-
9[c]	6	400	85	100	60	10	55	-
10[d]	6	400	85	100	60	10	55	-
11[e]	1	170	17	16	340	4	60	34
12[f]	1	170	17	16	340	4	60	34
13[g]	1	170	17	16	340	4	60	36
14[h]	1	170	17	16	340	4	60	23.5
15[i]	1	170	17	16	340	4	60	30

16[j]	1	170	17	16	340	4	60	37
17[k, l]	5	100	-	20	160	10	55	55
18[k, l]	5	100	25	20	160	10	55	43

The following footnotes describe the order of addition of reagents to ferrous ions in reaction vials, the buffer used, and any other relevant information specific to the experiments:

[a] added all reagents at once

[b] added phosphate ions, then sulfide ions and thioester

[c] added sulfide ions and thioester added, then phosphate ions

[d] added sulfide ions, then thioester (this mixture was stirred at 45°C for 1hr), then phosphate ions

[e] added sulfide ions, then phosphate ions, then thioester

[f] added sulfide ions and an equimolar amount of phosphate ions, then rest of phosphate ions, then thioester

[g] added phosphate ions, then sulfide ions, then thioester

[h] added thioester, then sulfide ions, then phosphate ions

[i] added thioester, then sulfide ions and an equimolar amount of phosphate ions, then rest of phosphate ions

[j] added thioester, then phosphate ions, then sulfide ions

[k] checked for pyrophosphate ions after 2, 4, 7 and 10 days

Table 5.3: Hydrolysis results for reactions 17 and 18 (presented in graph 2.2)

	% Hydrolysis of *N,S*-diacetyl cysteamine			
Reaction	After 2 days	After 4 days	After 7 days	After 10 days
17	16	29	42	55
18	15	24	33	43

5.4.7 Experiments from section 2.4.2

Reaction involving ferrous phosphate:

Solutions of *N,S*-diacetyl cysteamine (0.40M) in 1M PIPES (pH 6.8), imidazole (0.80M) in 1M PIPES (pH 6.8), ferrous sulfate (0.52M) in water and potassium dihydrogen phosphate (1.00M) in water were generated anaerobically. 0.25mL of each solution was transferred to degassed reaction vials; a white $FePO_4$ precipitate formed immediately. Reaction mixtures therefore contained 0.10M thioester, 0.20M imidazole, 0.43 ferrous ions, 0.25M phosphate ions and 0.5M PIPES (pH 6.6). Reaction mixtures were incubated in argon-filled chambers at room temperature or 65°C. At specified times, 400μL aliquots were removed and added to 0.16mL of a solution of potassium hydroxide (3M) in D_2O (this represented a 12-fold molar excess of cyanide ions over ferrous ions). The resulting solutions were shaken and left for 10-15min, then centrifuged at 15000rpm for 5min. The supernatants were analysed by ^{31}P and ^{1}H NMR. No pyrophosphate was detected in these trials, and the ^{1}H NMR results are summarised in table 5.4.

Table 5.4: Hydrolysis of *N,S*-diacetyl cysteamine when incubated with $FePO_4$ and imidazole

Incubation regime		
Time	Temperature	% Hydrolysis
4 days	room temperature	14
11 days	room temperature	26
17 hours	65°C	100

Reactions involving hydroxyapatite:

0.25mL of 0.4M potassium phosphate (pH 7.0), 0.25mL 1.0M imidazole hydrochloride (pH 7.0), and 0.25mL of 0.5M *N,S*-diacetyl cysteamine were added to 0.5mL of an aqueous suspension of hydroxyapatite (84mgmL^{-1}, 1.0M in phosphate), and incubated with stirring at room temperature or 50°C. Aliquots were periodically taken to assay for pyrophosphate and/or loss of thioester. To assay for thioester, a 0.2mL aliquot was added to 50μL D_2O, the solution centrifuged for 5min at 15000rpm, and the supernatant analysed by ^{1}H NMR. To assay for pyrophosphate, a 0.25mL aliquot was added to 3mL of a 0.125M EDTA solution and stirred for 1hr, during which time the white hydroxyapatite precipitate all but disappeared completely. 0.7mL of this solution was added to 0.1mL D_2O and centrifuged for 5min at 15000rpm, before analysing by ^{31}P NMR.

Table 5.5: Hydrolysis of *N,S*-diacetyl cysteamine when incubated with hydroxyapatite and imidazole

Incubation regime		
Time	Temperature	% Hydrolysis
3 days	room temperature	0
6 days	room temperature	5
1 day	50°C	5
3 days	50°C	34
6 days	50°C	47

5.4.8 Experimental from section 2.5

A general experimental protocol is outlined below, with specific details being given in table (ie, reagent stoichiometries, order of addition of reagents during generation of reaction mixtures, the buffer used (500mM) and temperature and duration of

incubations). Reactions are numbered in chronological order from 1-15 for ease of reference

Solutions of ferrous sulfate in water, sodium hydrogen sulfide in water, potassium dihydrogen phosphate in 1M MES (pH 6.5) and thioacetic acid in 1M MES (pH 6.5) were generated anaerobically. Generally, the stoichiometries of these reagent solutions allowed 0.25mL of each to be transferred to reaction vials to give 1mL reaction mixtures at the desired stoichiometry. Occasionally larger volumes were used to allow several aliquots to be taken over the course of the reactions. When solutions of ferrous ions were mixed with solutions of sulfide ions or phosphate ions black FeS precipitates and white $FePO_4$ precipitates, respectively, formed immediately. In control experiments water was added in place of the ferrous ion or sulfide solutions. All reaction mixtures were incubated in argon filled chambers. At the end of an incubation, a 12-fold molar excess (relative to ferrous ion concentration) of potassium cyanide was added as a 3M aqueous solution. The solutions were shaken and left to stand for 10-15min, then centrifuged at 15000rpm for 5min. 0.7mL of the supernatants were mixed with 0.1mL aliquots of D_2O and analysed by ^1H and ^{31}P NMR. ^1H NMR was used to assay for loss of thioester or thioacid; the large water peak was calibrated to δ 4.60ppm, and the acetic acid and thioacetic acid gave clear signals at δ_H 1.83 and 2.36ppm, respectively. ^{31}P NMR was used to assay for pyrophosphate production. The large phosphate signal was calibrated to 3.37ppm, and pyrophosphate signals appeared around –5.5ppm (the chemical shifts varied with pH). When necessary, samples were spiked with pyrophosphate either directly, or by the inclusion of an NMR tube-insert containing a pyrophosphate solution during analysis.

No pyrophosphate production was detected in any of the reaction mixtures.

Table5.6: A summary of experiments attempting to detect pyrophosphate production with thioacetic acid as dehydrating agent

			Reagent concentrations (mM)				Incubation regimes		
# and notes	Volume	Buffer	Thioacid	Ferrous ions	Sulfide ions	Phosphate ions	Time (days)	Temperature (°C)	hydrolysis
1[a]	1.2	PIPES (pH 6.8)	875	85	80	65	2	45	-
2[b]	1.2	PIPES (pH 6.8)	875	85	80	65	2	45	-
3[c]	1.2	PIPES (pH 6.8)	875	85	80	65	2	45	35
4[d, e]	1.5	MES (pH 6.5)	130	20	10	10	8	50	49
5[d, f]	1.5	MES (pH 6.5)	130	20	-	20	8	50	55
6[g]	5	Bis-TRIS (pH 6.5)	160	25	25	13	4	55	48
7[h]	5	MES (pH 6.5)	100	25	20	160	8	50	26
8[b]	2	PIPES (pH 6.8)	190	40	20	20	1	55	48
9[a]	2	Bis-TRIS (pH 6.5)	25	10	5	5	1	58	65
10[i]	2	Bis-TRIS (pH 6.5)	25	10	5	5	1	58	61
11[j]	1	PIPES (pH 6.8)	50	20	5	16	1	58	25
12[j]	1	PIPES (pH 6.8)	50	20	24	0	1	58	50
13[k, l]	2	Bis-TRIS (pH 6.5)	50	10	-	10	20	65	19
14[b]	2	Bis-TRIS (pH 6.5)	50	10	2.5	100	20	65	14
15[b]	2	Bis-TRIS (pH 6.5)	50	10	2.5	7.5	20	65	19

16[b, m]	5	MES(pH 6.5)	125	30	25	200	10	50	24

The following footnotes describe the order of addition of reagents to ferrous ions in reaction vials, the buffer used, and any other relevant information specific to the experiments:

[a] added sulfide ions, then thioacid (incubated at 45°C for 30min), then phosphate ions

[b] added thioacid, then sulfide ions, then phosphate ions

[c] all reagents added at once

[d] checked for pyrophosphate after 1,3 and 8 days

[e] added thioacid and sulfide ions, then phosphate ions

[f] added thioacid and phosphate ions, then sulfide ions

[g] reagents (including ferrous sulfate) weighed together and dissolved in 500M Bis-TRIS ; checked for pyrophosphate after 2 and 4 days

[h] added thioacid, then sulfide and an equimolar amount of phosphate, then rest of phosphate; checked for pyrophosphate after 2, 4, 7 and 10 days

[i] added thioacid, then sulfide ions and phosphate ions

[j] the order of addition of reagents was not recorded

[k] added thioacid, then phosphate ions; made up to volume with buffer solution

[l] made up to volume with water and/or buffer solutions

[m] checked for pyrophosphate after 2, 4, 7 and 10 days

5.4.9 Experimental from section 2.6

Solutions of ferrous sulfate (50mM) in water, sodium hydrogen sulfide (150mM) in water, potassium dihydrogen phosphate (150mM) in water and thioacetic acid (50mM) in 1M Bis-TRIS (pH 6.5) were generated anaerobically. To degassed reaction vials was transferred 0.25mL of the ferrous ion solution, 0.50 mL of the thioacid solution and 0.25mL of either the sulfide ion or phosphate ion solution. Black FeS precipitates and white $FePO_4$ precipitates formed immediately in sulfide ion- and phosphate ion-containing reaction mixtures, respectively. Two control solutions were generated: in the first 0.25mL water was added in place of the sulfide and phosphate solutions; and in the second 0.5mL of the thioacid solution was mixed with 0.50mL of water. Reaction mixtures were incubated in argon-filled chambers at 65°C for 17hr. At the end of the incubation period 100μL aliquots of a solution potassium cyanide (3M) in D_2O were added to each reaction mixture. The resulting solutions were shaken and left to stand for 10-15min, then centrifuged at 15000rpm for 5min. The supernatants were analysed by ^1H NMR. See table 4. for results

5.5 Experimental from chapter 3

5.5.1 *N,S*-Diacetyl cysteamine redox experiments

2,4-Dinitrophenylhydrazine solution:

2,4-dinitrophenylhydrazine (2,4-DNP) was recrystallised from ethanol. 0.5g of this pure 2,4-DNP was weighed into a 100mL round bottomed flask. 100mL of methanol was added, and, with vigorous stirring, 1mL of concentrated sulfuric acid was added dropwise. The crystals dissolved to give an orange solution, which was filtered.

Assaying for acetaldehyde:

Manipulations were carried out in a "cold room", at 4°C. Solutions to be assayed were removed from the incubator and left at 4°C for 30min. 0.1mL of a 2,4-DNP solution ($2.5x10^{-5}$M) was then added, and the solution incubated at 45°C for 10min. 0.5mL of ether was added and shaken with the solution. The ether was allowed to

separate from the aqueous phase, and was then spotted directly onto a TLC plate. A 1:2 pet ether/ethyl acetate mixture was used as the eluant solution. Acetaldehyde hydrazone migrated with an R_f value of 0.91, while the unreacted hydrazine, which was also extracted by the ether, migrated with an R_f value of 0.63.

Determining the detection limit of acetaldehyde:

Qualitative analysis of acetaldehyde was carried out by comparing the relative intensities of the hydrazine and hydrazone spots on TLC. To three 1mL aliquots of a $2.5x10^{-3}$M acetaldehyde solution was added 0.1mL of $2.5x10^{-2}$M, $1.25x10^{-2}$M, and a $2.5x10^{-3}$M 2,4-DNP solutions. The three solutions were analysed as described above. When the ratio of acetaldehyde to 2,4-DNP was 1:1 the hydrazine spot was darker than the hydrazone spot, when the ratio was 2:1 the intensity of the two spots was approximately equal, and when the ratio was 10:1 the hydrazone spot dominated, with only a weak hydrazine signal. It was therefore concluded that a 10-fold excess of acetaldehyde over 2,4-DNP was necessary for definitive results.

To three 1mL aqueous acetaldehyde solutions with concentrations of $2.5x10^{-3}$M, $2.5x10^{-4}$M and $2.5x10^{-5}$M was added 0.1mL of 2,4-DNP solutions of the same concentration. The solutions were analysed as outlined above. The experiment containing the highest concentration of hydrazine solution gave a dark hydrazone spot and a much lighter hydrazine spot, the $2.5x10^{-4}$M acetaldehyde trial gave similarly unambiguous results, although the spots were much fainter, and the experiment at lowest hydrazine concentration showed no discernible spots. The detection limit of acetaldehyde in aqueous solutions was therefore around $2.5x10^{-4}$M.

Reduction experiments:

All manipulations involving sample preparation were carried out anaerobically. Solutions of ferrous sulfate (200mM) in 1M PIPES (pH 6.8) and *N,S*-diacetyl cysteamine (50mM) in water were generated. 0.25mL aliquots of each solution were transferred to reaction vials, to give 0.5mL reaction solutions that contained 25mM thioester, 100mM ferrous ions and 500mM PIPES buffer. Blanks were generated with 1M PIPES solution in the place of ferrous sulfate solution. Reaction vials were

incubated under argon for 20hr at 45°C. Analysis of solutions was carried out as outlined in section 3.1.4.

5.5.2 *N,S*-Dibenzoylcysteamine redox experiments

Synthesis of N,S-dibenzoylcysteamine[4]*:*

1.93g (25mmol) of cysteamine was weighed into a 3-necked round-bottomed flask. A magnetic flea was included, and the flask placed over a magnetic stirrer in an ice bath. 40mL of water was added and the cysteamine was dissolved with stirring. A pH electrode was fitted into one neck and a 50mL dispensing column containing benzoyl chloride (11.04mL, 95mmol) into another. The benzoyl chloride was added dropwise over several minutes with the simultaneous addition of a 2M potassium hydroxide solution to keep the pH between 7.5 and 8. The product was a white viscous oil however, which quickly collected around the pH electrode, leaving the pH readings ambiguous. Around 30mL of potassium hydroxide was added in total. By the end of the addition 2 layers were seen in the reaction vial; an upper colourless solution and a lower milky-white liquid. The pH readings obtained from these two phases were 11 and 7.5, respectively. Concentrated hydrochloric acid was added to bring the pH of the upper phase to 7.5. Upon removal of the flask from the ice bath it was observed that the white liquid had solidified to become an off-white non-crystalline solid. The solution was saturated with sodium chloride, and then extracted with dichloromethane (3x20mL); the white solid was extracted. The dichloromethane solution was dried with magnesium sulfate, filtered, and the solvent removed using a rotary evaporator to leave a yellow oil of low viscosity. The product was purified from this crude mixture by flash chromatography. Silica gel was used as the solid matrix, and a 1:1 mixture of ethyl acetate and petroleum ether as the eluant. 4g of the crude product was placed on the column, and ten 30mL fractions were taken. Fractions 4-6 were shown by thin layer chromatography to contain the desired product, so these fractions were combined and the solvent removed using a rotary evaporator to leave white crystals.

Yield: 2.5g (35%)

^{1}H NMR ($CDCl_3$): δ 3.32 (t, 2H, SC**H**$_2$), 3.72 (q, 2H, NHC**H**$_2$), 7.03 (bs, 1H, N**H**), 7.38-7.98 (m, 10H, 2xAr**H**)

^{13}C NMR ($CDCl_3$): δ 28.42 (SCH_2), 40.29 ($NHCH_2$), 126.92 (Ar), 127.22 (Ar), 128.42 (Ar), 128.61 (Ar), 131.41 (Ar), 133.64 (Ar), 134.05 (Ar), 136.55 (Ar), 167.63 (CO), 192.60 (CO)

Reduction experiments:

All manipulations involving sample preparation were carried out anaerobically. Solutions of ferrous sulfate (17mM) in water and *N,S*-dibenzoylcysteamine (5mM) in methanol were generated. 2.1mL of the ferrous solution and 3.5mL of the thioester solution were transferred to reaction vials. The reaction solutions therefore contained 6.4mM ferrous ions and 3.1mM thioester in an unbuffered aqueous 60% methanol solvent. The pH of these solutions, as determined by pH paper, was around 5. Blanks were generated by substituting water for the ferrous solution, and spiked samples were spiked with distilled benzaldehyde in methanol using a 10μL Hamiltonian syringe. Solutions were incubated at 60°C in argon-filled chambers. The solutions were removed from the incubating chambers after 24hrs, and a 12-fold molar excess (relative to ferrous ion concentration) of potassium cyanide was added as a 6M aqueous solution. The solutions were centrifuged at 15000rpm for 5min, and the supernatant transferred to a 25mL volumetric flask. Ether, which was to be used in sample extractions, was miscible with the 60% methanol reaction solvent, so to make extractions possible, the 5.6mL supernatants were first diluted with 12mL of water; and 3mL of ether was then used to extract this solution. The ether was transferred to a vial containing a small spatula load of anhydrous magnesium sulfate, and was stirred for a few minutes before being filtered into a clean vial. This solution was spotted ten times onto a TLC plate (silica on aluminium) and run in a 1:1 ethyl acetate/pet ether eluant. The plate was then placed under a UV lamp for analysis. Reference samples of benzoic acid, benzaldehyde and benzyl alcohol were run in parallel.

5.5.3 *N*-Acetyl, *S*-benzoyl redox experiments

Synthesis of N-acetyl cysteamine[2]:

1.53g of *N,S*-diacetyl cysteamine (9.5mmol) was weighed into a 100mL flask. A magnetic flea was included and the flask placed in an ice bath on a magnetic stirrer. 50mL of water was added and the *N,S*-diacetyl cysteamine was dissolved with stirring. Solid potassium hydroxide was added (1.77g, 31.6mmol, 3 mol. equiv.), the flask degassed and placed under argon, and the solution stirred at room temperature

for thirty minutes. Hydrochloric acid (2M) was added until the pH reached 7. The solution was then saturated with sodium chloride and extracted with dichloromethane (3x20mL). The dichloromethane solution was dried by stirring with magnesium sulfate, filtered, and the solvent removed with a rotary evaporator to leave a colourless oil. ^{1}H NMR showed this oil to be a pure sample of the desired product.

Yield: 0.79g (70%)

^{1}H NMR ($CDCl_3$): δ 1.35 (t, 1H, S**H**), 1.99 (NHCOC**H**$_3$), 2.66 (q, 2H, SHC**H**$_2$), 3.41 (q, 2H, NHC**H**$_2$), 6.03 (bs, 1H, N**H**)

Synthesis of N-acetyl, S-benzoyl cysteamine[4]*:*

0.78g (6.63mmol) of *N*-acetyl cysteamine was dissolved in 30mL of water in a 100mL round-bottomed flask. A magnetic flea was included, and the flask placed in an ice bath on a magnetic stirrer. A pH meter was used to measure the pH, which was just below neutrality. A 2M potassium hydroxide solution was added to lift the pH to 9. 1.3g of benzoyl chloride (9.25mmol, 1.4mol. equiv.) was added drop-wise over 5 minutes, with the simultaneous addition of 2M potassium hydroxide solution to keep the pH between 9 and 9.5. A white precipitate appeared and collected around the pH meter during the addition, so the pH of the solution at times could have climbed above 9.5. The solution was left to stir on ice for a further one hour with intermittent additions of potassium hydroxide to keep the pH above 9. The solution was then saturated with sodium chloride and extracted with ethyl acetate (3x20mL); the white precipitate was extracted. The dichloromethane solution was dried with magnesium sulfate, filtered, and the solvent removed using a rotary evaporator. An off-white oil remained, which was placed under a high vacuum to remove dissolved dichloromethane. A ^{1}H NMR of this sample showed it to be contaminated with benzoic acid. The oil crystallised after about thirty minutes at room temperature, and was recrystallised from ethyl acetate with petroleum ether. A ^{1}H NMR showed that the desired product had been successfully separated from the benzoic acid contaminant.

Yield: 0.766g (52%)

^{1}H NMR ($CDCl_3$): δ 1.96 (s, 3H, C**H**$_3$), 3.22 (t, 2H, SC**H**$_2$), 3.52 (q, 2H, NHC**H**$_2$), 5.98 (bs, 1H, N**H**), 7.45 (t, 2H, m-Ar**H**), 7.56 (t, 1H, p-Ar**H**), 7.96 (d, 2H, o-Ar**H**)

^{13}C NMR ($CDCl_3$): δ 23.13 (S**C**H$_2$), 28.48 (CO**C**H$_3$), 39.59 (NH**C**H$_2$), 127.21 (Ar), 128.63 (Ar), 133.63 (Ar), 136.59 (Ar), 170.43 (**C**O), 192.15 (**C**O)

Reduction experiments

All manipulations involving sample preparation were carried out anaerobically. Solutions of ferrous sulfate (80mM) in water, sodium hydrogen sulfide (160mM) in water and *N*-acetyl, *S*-benzoyl cysteamine (20mM) in a 10% acetonitrile/1M PIPES solution were generated. 0.25mL of each of the first two solutions and 0.5mL of one of the latter solutions were transferred to degassed reaction vials, to give reaction solutions that contained 20mM ferrous ions, 40mM sulfide ions, 10mM thioester and 500mM PIPES in an aqueous solution containing 5% acetonitrile. Blanks were included in each run, which contained 0.25mL of water in the place of the ferrous sulfate solution. The vials were incubated in argon-filled chambers for 5 days at 68°C. At the end of the incubation period, a twelve-fold excess (relative to ferrous ion concentrations) of potassium cyanide was added to all solutions, including the blanks. An orange-red iron cyanide complex immediately formed in iron-containing solutions. Solutions were centrifuged at 15000rpm for 5min, to give a dark coloured pellet, and a supernatant fraction free from precipitate. A C_{18} cartridge was used to prepare samples for analysis by HPLC (see section 5.1.3). HPLC analysis involved an acetonitrile/water gradient, where the acetonitrile concentration was increased from 30% to 60% over 20min. Using this solvent system, standard samples of benzyl alcohol, benzoic acid, *N*-acetyl, *S*-benzoyl cysteamine, benzaldehyde and thiobenzoic acid eluted from the column after 6min, 7.5min, 9.5min, 11.5min and 15.5min, respectively.

5.5.4 Thiobenzoic acid redox experiments

Synthesis of thiobenzoic acid[5]*:*

Potassium hydroxide (20g, 357mmol) was weighed into a 3-necked 250mL round-bottomed flask. A flea was added, suba seals fitted, and the flask and contents degassed. 80mL of degassed 90% ethanol was added to dissolve the potassium

hydroxide, and the flask placed in an ice bath over a magnetic stirrer. The flask was placed under a positive pressure of argon and hydrogen sulfide was bubbled through the solution with vigorous stirring. The solution went from colourless to maroon to colourless to green/blue. At this time the pH, as determined by pH paper was around 9.5. Benzoyl chloride (2.3g, 14mmol) was added dropwise with stirring and cooling over 70min. A white precipitate formed and the solution went a yellow/blue colour. This solution was stirred with cooling for a further 2hr. The solution was filtered and the crystals washed with 20mL 95% ethanol. The filtrate was placed on a rotary evaporator to remove the solvent, and the resulting residue dissolved in 100mL water to give a murky orange/yellow solution and a few mL of a dark green immiscible liquid. Potassium hydroxide was added to raise the pH of the solution from <5 to >9. The dark green immiscible liquid solidified with this basification. The solution was filtered, the filtrate washed with 50mL pet ether, and then acidified with cold 6M hydrochloric acid. The yellow solution became murky and a green oil separated. This solution was extracted twice with 40mL peroxide-free pet ether*; the first extraction was dark green and the second was intense yellow/orange. Both extractions were pooled and the ether washed twice with 30mL water, dried with anhydrous sodium sulfate, filtered, and placed on a rotary evaporator at room temperature over about 1hr. The ether solvent was removed to leave a yellow oil (thiobenzoic acid), which was then distilled under argon. Thiobenzoic acid boiled at a temperature of 62-63°C. 1mL fractions were collected; the first was light yellow in colour, but the next two became progressively darker. Only the first fraction was used in experiments.

^{1}H NMR ($CDCl_3$): δ 4.58 (bs, 1H, S**H**), 7.46 (t, 2H, m-Ar**H**), 7.57 (t, 1H, p-Ar**H**), 7.89 (d, 2H, 0-Ar**H**)

Reduction experiments:

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* Peroxide-free pet ether was prepared immediately prior to use by washing ether twice with a slightly acidic ferrous solution followed by one wash with deionised water.

All manipulations involving sample preparation were carried out anaerobically. Solutions of ferrous sulfate (80mM) in water, sodium hydrogen sulfide (160mM) in water, and of *N*-acetyl-*S*-benzoylcysteamine (20mM) or thiobenzoic acid (20mM) in 1M PIPES were generated. The thioester solution contained 10% v/v acetonitrile. 0.25mL of each of the first two solutions and 0.5mL of one of the latter solutions were transferred to degassed reaction vials, to give reaction solutions that contained 20mM ferrous ions, 40mM sulfide ions, and 10mM thioester or thioacid. Blanks were also included in each run, which contained 0.25mL of water in the place of the ferrous sulfate solution. The vials were incubated in argon-filled chambers for 5 days at 68°C. At the end of the incubation period, a twelve-fold excess (relative to ferrous ion concentrations) of potassium cyanide was added to all solutions, including the blanks. An orange-red iron cyanide complex immediately formed in iron-containing solutions. Solutions were centrifuged at 15000rpm for 5min, to give a dark coloured pellet, and a supernatant fraction free from precipitate. A C_{18} cartridge was used to prepare samples for analysis by HPLC (see section 5.1.3). HPLC analysis involved an acetonitrile/water gradient, where the acetonitrile concentration was increased from 30% to 60% over 20min. Using this solvent system, standard samples of benzyl alcohol, benzoic acid, *N*-acetyl, *S*-benzoyl cysteamine, benzaldehyde and thiobenzoic acid eluted from the column after 6min, 7.5min, 9.5min, 11.5min and 15.5min, respectively. Samples to be analysed by HPLC/MS were also analysed as acetonitrile solutions.

5.6 Experimental from chapter 4

5.6.1 Synthesis of Z-amino acids[6]

The amino acid (0.05mol) was dissolved in 25mL of 4M sodium hydroxide in a 100mL round-bottomed flask, and the resulting solution cooled in an ice bath. Benzyl chloroformate (0.06 mol) was added in 10 aliquots every 10min with stirring and cooling. The temperature remained between 0 and 5°C and the pH above 11 throughout the addition. After all the benzyl chloroformate had been added the solution was left to stir at room temperature for 1hr. The solution was then extracted with 2x 20mL ether. Acidification with conc. HCl gave a white precipitate which was

filtered, washed with water and air dried in a vacuum dessicator overnight at 80°C. This crude product was recrystallized from chloroform with pet ether to give white needle-shaped crystals.

Z-L-alanine

Yield: 1.6g (14%)

m.p.: 110-112°C

^{1}H NMR (d_6-DMSO): δ 1.37 (d, 3H, C**H**$_3$), 4.13 (m, 1H, NHC**H**), 5.13 (s, 2H, C**H**$_2$), 7.45 (m, 5H, Ar**H**), 7.72 (d, 1H, N**H**)

^{13}C NMR (d_6-DMSO): δ 17.25 (**C**H$_3$), 49.42 (NH**C**H), 65.55 (**C**H2), 127.92, 127.98, 128.51, 137.20 (Ar), 156.04 (**C**O), 174.60 (**C**O)

Z-glycine

Yield: 2.6g (26%)

m.p.: 118-120°C

^{1}H NMR (d_6-DMSO): δ 3.79 (d, 2H, NHC**H**$_2$), 5.15 (s, 1H, ArC**H**$_2$), 7.46 (m, 5H, Ar**H**), 7.68 (t, 1H, N**H**)

^{13}C NMR (d_6-DMSO): δ 42.34 (NH**C**H$_2$), 65.68 (Ar**C**H$_2$), 127.91 (Ar), 128.02 (Ar), 128.54 (Ar), 137.20 (Ar), 156.73 (**C**O), 171.80 (**C**O)

5.6.2 Synthesis of *N*-hydroxysuccinimide[7]

Succinic anhydride (100g, 1.0mol) and hydroxylamine hydrochloride (70g, 1.0mol) were combined in a 500mL round-bottomed flask. The flask was placed on a rotary evaporator with a liquid nitrogen cold-trap, and heated by a silicone oil bath. The contents of the flask were rapidly heated to 125°C, at which point fusion began to

occur with the evolution of gas. The temperature was increased slowly to 160°C over the next hour. After a further hour at this temperature the formation of water had ceased, and the heating was discontinued. When the temperature had dropped to 125°C the amber liquid was poured into a beaker containing 400mL of ether. The ether was stirred vigorously during the addition, and the crude product solidified shortly after. The ether was decanted and 400mL of dry 1-butanol was added to the solid residue The resulting solution was heated to boiling to dissolve the crude product. This solution was filtered and rapidly chilled to 0°C, then left in the freezer overnight. The next day fine white crystals were filtered from the solution (45g), and washed once with 1-butanol (50mL) and once with ether (50mL). More crystals were recovered from the 1-butanol supernatant and washed in a similar manner (8g). The product was recrystallised from ethyl acetate.

Yield: 40g (35%)

m.p.: 95-97°C

^{1}H NMR (D_2O): δ 2.67 (s, 2xCH_2)

^{13}C NMR (D_2O): δ 26.08 (CH_2), 177.25 (CO)

5.6.3 Synthesis of *N*-hydroxysuccinimide esters of Z-amino acids[7]

N-hydroxysuccinimide (2.5g, 21.7mmol) and Z-amino acid (21.7mmol) were combined in a 100mL round-bottomed flask. A magnetic stirring flea was also included, and the flask was placed in an ice bath. The contents were dissolved in dry dioxane (50mL), with stirring and cooled to <5°C. DCC (4.48g, 21.7mmol) was added, at which point a suba-seal was attached and the contents of the flask placed under argon. The solution was stirred for several hours in the ice-bath, during which time a white precipitate formed (dicyclohexylurea). The flask was left in the fridge at 4°C overnight. The following day the solution was filtered and the whit precipitate washed with dry dioxane (2x5mL). The filtrate and washings were combined, and the dioxane removed using a rotary evaporator. A yellow oil was left, which gave

crystals after several hours in the fridge. This was then recrystallized from isopropanol to give white needle-shaped crystals.

The *N*-hydroxysuccinimide ester of Z-L-alanine

Yield: 4.98g (72%)

m.p.: 102-104°C

^{1}H NMR ($CDCl_3$): δ 1.58 (d, 3H, C**H**$_3$), 2.80 (s, 4H, C**H**$_2$C**H**$_2$), 4.76 (m, 1H, NHC**H**), 5.12 (s, 2H, ArC**H**$_2$), 5.51 (d, 1H, N**H**), 7.35 (m, 5H, Ar**H**)

^{13}C NMR ($CDCl_3$): δ 18.47 (**C**H$_3$), 25.45 (2x**C**H$_2$), 48.01 (NH**C**H), 67.22 (Ar**C**H$_2$), 128.14 (Ar), 128.21 (Ar), 128.48 (Ar), 135.91 (Ar), 155.63 (**C**O), 168.72 (2x**C**O), 170.72 (**C**O)

The *N*-hydroxysuccinimide ester of Z-glycine

Yield: 5.96g (90%)

m.p.: 111-113°C

^{1}H NMR ($CDCl_3$): δ 2.78 (s, 4H, C**H**$_2$ C**H**$_2$), 4.30 (d, 2H, NHC**H**$_2$), 5.12 (s, 2H, ArC**H**$_2$), 5.67 (t, 1H, N**H**), 7.34 (m, 5H, Ar**H**)

^{13}C NMR ($CDCl_3$): δ 25.25 (**C**H$_2$CH$_2$), 25.44 (CH$_2$**C**H$_2$), 40.53 (NH**C**H$_2$), 67.33 (Ar**C**H$_2$), 128.11 (Ar), 128.21 (Ar), 128.47 (Ar), 135.91 (Ar), 156.17 (NH**C**O), 166.06 (**C**O), 168.98 (**C**O)

5.6.4 Synthesis of Z-amino thioacids[8]

Z-Amino acid-*N*-hydroxysuccinimide ester (1mol equiv.) was dissolved in dry DMF (10mL for every 1g of ester used) with stirring and cooling with an ice bath. Li_2S

(2mol equiv.) was added resulting in a turquoise-coloured solution. This solution was stirred for an hour, during which time the colour changed to yellow. It was then acidified with 3% HCl. During the acidification the solution first became intensely yellow and finally a cloudy white precipitate formed. The flask was removed from the ice-bath, and the crude product extracted with ethyl acetate, which was washed twice with water. The ethyl acetate was removed using a rotary evaporator, leaving a yellow oil. This was extracted repeatedly with hot pet ether. Crystals formed upon cooling and were filtered from the solution. Further thioacid was obtained by placing the filtrate on a rotary evaporator and removing about half of the pet ether.

Z-L-alanine thioacid

Yield: 34% (0.41g)

^{1}H NMR ($CDCl_3$): δ 1.40 (d, 3H, C**H**$_3$), 4.42 (m, 1H, NHC**H**), 5.14 (s, 2H, ArC**H**$_2$), 5.41 (d, 1H, N**H**), 7.35 (m, 5H, Ar**H**)

Z-glycine thioacid

Yield: 33% (1.21g)

m.p.: 72-74°C

^{1}H NMR ($CDCl_3$): δ 4.12 (d, 2H, NHC**H**$_2$), 5.15 (s, 2H, ArC**H**$_2$), 5.45 (s, 1H, N**H**), 7.36 (7.37 (m, 5H, Ar**H**)

^{13}C NMR ($CDCl_3$): δ 51.70 (NH**C**H$_2$), 67.29 (Ar**C**H$_2$), 128.01 (Ar), 128.21 (Ar), 128.46 (Ar), 135.83 (Ar), 156.24 (NH**C**O), 196.54 (**C**OSH)

5.6.5 Synthesis of potassium salts of Z-amino thioacids[8]

The Z-amino thioacid was dissolved in 1M potassium hydroxide to give a solution with a pH of 7. The water was removed by freeze-drying, and the product recrystallised from ethyl acetate with pet ether.

Potassium salt of Z-L-alanine thioacid

Yield: 2.7g (60%)

m.p.: 100-101°C

^{1}H NMR (D_2O): δ 1.24 (d, 3H, C**H**$_3$), 4.18 (q, 1H, NHC**H**), 4.97 (m, 2H, ArC**H**$_2$), 7.30 (m, 5H, Ar**H**)

^{13}C NMR (D_2O): δ 19.94 (**C**H$_3$), 61.87 (NH**C**H), 67.40 (Ar**C**H$_2$), 128.53 (Ar), 129.16 (Ar), 129.62 (Ar), 137.34 (Ar)

Potassium salt of Z-glycine thioacid

Yield: 27% (0.40g)

^{1}H NMR (D_2O): δ 3.93 (s, 2H, NHC**H**$_2$), 4.95 (s, 2H, ArC**H**$_2$), 7.28 (m, 5H, Ar**H**)

^{13}C NMR (D_2O): δ 54.39 (NH**C**H$_2$), 67.00 (Ar**C**H$_2$), 127.75 (Ar), 128.35 (Ar), 128.76 (Ar), 136.38 (Ar), 158.34 (NH**C**O), 217.12 (**C**OSH)

5.6.6 Synthesis of amino acid methyl ester hydrochloride salts[9]

The amino acid was dissolved in methanol, and stirred in an ice bath. Thionyl chloride was added drop-wise. The solution was left to stir at room temperature for 48hr. The methanol was removed using a rotary evaporator, and the remaining crystals were washed twice with ether and recrystallised with methanol/ether.

The hydrochloride salt of L-alanine methyl ester

Yield: 1.33g (82%)

m.p.: 156-158°C

^{1}H NMR (d_6-DMSO): δ 1.50 (d, 3H, CHC**H**$_3$), 3.79 (s, 3H, OC**H**$_3$), 4.10 (q, 1H, C**H**), 8.87 (s, 3H, N**H**$_3^+$)

^{13}C NMR (d_6-DMSO): δ 15.74 (CH**C**H$_3$), 47.93 (**C**H), 52.89 (O**C**H$_3$), 170.43 (**C**O)

The hydrochloride salt of glycine methyl ester

Yield: 1.34g (92%)

m.p.: 175°C

^{1}H NMR (d_6-DMSO): δ 3.79 (s, 3H, C**H**$_3$), 3.83 (s, 2H, C**H**$_2$), 8.77 (s, 3H, N**H**$_3^+$)

^{13}C NMR (d_6-DMSO): δ 39.60 (**C**H$_2$), 52.68 (**C**H$_3$), 168.12 (**C**O)

The hydrochloride salt of L-phenylalanine methyl ester

Yield: 2.18g (87%)

m.p.: 158-162°C

^{1}H NMR (D_2O): δ 3.19 (o, 2H, C**H**$_2$), 3.75 (s, 3H, C**H**$_3$), 4.34 (t, 1H, C**H**), 7.27 (m, 5H, Ar**H**)

^{13}C NMR (d_6-DMSO): δ 35.91 (**C**H$_2$), 52.60 (**C**H$_3$), 53.46 (**C**H), 127.35 (Ar), 128.69 (Ar), 129.51 (Ar), 134.96 (Ar), 169.40 (**C**O)

5.6.7 Synthesis of Z-amino acyl-amino acid methyl ester[10]

A Z-amino acid (2.25mmol) and an amino acid methyl ester (2.25mmol) were weighed into a 50mL round bottomed flask and dissolved in dichloromethane (20mL). The flask was placed in an ice bath and the solution stirred with a magnetic flea. 1-hydroxybenzotriazole (0.39g, 2.91mmol, 1.3mol equiv), diisopropylethylamine

(0.237mL, 2.46mmol, 1.1mol equiv) and dicyclohexylcarbodiimide (0.69g, 3.36mmol, 1.5mol equiv.) were added in that order. The flask was fitted with a suba seal, and the contents placed under a positive pressure of nitrogen. The solution was left to stir in the ice bath for a further 1hr, during which time a white precipitate of dicyclohexylurea formed. The solution was left to stir at room temperature overnight. The next day the solution was filtered and the precipitate washed with dichloromethane (10mL). This filtrate was washed with a saturated aqueous solution of sodium bicarbonate (20mL) followed by a saturated brine solution (20mL). The dichloromethane was dried with magnesium sulfate before being removed using a rotary evaporator, to leave the crude product. The product was purified by flash chromatography, then recrystallised from ethyl acetate with pet ether to give white needle crystals.

Z-L-alanyl L-alanine methyl ester

Yield: 0.24g (35%)

m.p.: 71-73°C

^{1}H NMR ($CDCl_3$): δ 1.37 (d, 6H, 2xCHC**H**$_3$), 3.72 (s, 3H, OC**H**$_3$), 4.12 (q, 1H, OCOC**H**), 4.55 (q, 1H, NCOC**H**), 5.10 (s, 2H, C**H**$_2$), 5.39 (d, 1H, N**H**COO), 6.61 (d, 1H, N**H**COCH), 7.33 (m, 5H, Ar**H**)

^{13}C NMR ($CDCl_3$): 18.05 (OCOCH**C**H$_3$), 18.09 (OCONHCH**C**H$_3$), 48.05 (OCO**C**H), 50.34 (OCONH**C**H), 52.47 (O**C**H$_3$), 67.02 (**C**H$_2$), 128.01 (Ar), 128.14 (Ar), 128.47 (Ar), 136.21 (Ar), 155.87 (**C**O), 172.01 (**C**O), 173.16 (**C**O)

Z-L-alanyl glycine methyl ester

Yield: 0.25g (36%)

m.p.: 72-74°C

^{1}H NMR ($CDCl_3$): δ 1.40 (d, 3H, $CHCH_3$), 3.72 (s, 3H, OCH_3), 4.00 (d, 2H, $NHCH_2$), 4.12 (q, 1H, $CHCH_3$), 5.10 (q, 2H, $ArCH_2$), 7.33 (m, 5H, ArH)

^{13}C NMR ($CDCl_3$): δ 18.38 ($CHCH_3$), 40.96 ($NHCH_2$), 52.18 ($CHCH_3$), 55.59 (OCH_3), 66.88 ($ArCH_2$), 127.85 (Ar), 128.02 (Ar), 128.36(Ar), 136.03 (Ar), 156.01 (CO), 170.02 (CO), 172.84 (CO)

Z-L-alanyl L-phenylalanine methyl ester

Yield: 0.29g (33%)

m.p.: 72-74°C

^{1}H NMR ($CDCl_3$): δ 1.28 (d, 2H, $CHCH_3$), 3.09 (o, 2H, $CHCH_2$), 3.69 (s, 3H, OCH_3), 4.25 (t, 1H, CH_2CH), 4.85 (p, CH_3CH), 5.09 (s, 2H, $ArCH_2$), 7.07-7.41 (m, 10H, 2xArH)

^{13}C NMR ($CDCl_3$): δ 18.62 ($CHCH_3$), 37.68 ($CHCH_2$), 50.38 ($CHCH_3$), 50.32 (OCH_3), 53.10 (CH_2CH), 66.93 ($ArCH_2$), 116.86-129.45 (8 signals, 2xAr), 155.85 (CO), 171.61 (CO), 172.13 (CO)

5.6.8 Synthesis of *N*-acetyl L-phenylalanine

L-Phenylalanine (0.826g, 5mmol) was weighed into a 100mL beaker and 30mL of water was added. A magnetic flea was included and the beaker placed into an ice bath over a magnetic stirrer. A pH electrode was fitted, and the pH adjusted to 10 with 8M potassium hydroxide. When the phenylalanine had completely dissolved, acetic anhydride (1.544g, 15mmol) was added drop-wise over 15 minutes, with concomitant addition of 8M potassium hydroxide to maintain the pH at around 10. The solution was stirred for a further 15 minutes to allow the pH to stabilise. Concentrated hydrochloric acid was added drop-wise until a white precipitate fell out of solution. This was extracted with ethyl acetate (2x30mL), which was then dried with magnesium sulfate and filtered. The solvent was removed using a rotary

evaporator to leave a white solid. This was recrystallised from ethyl acetate with pet ether to give white needle crystals.

Yield: 0.75g (72%)

m.p.: 67-70°C

^{1}H NMR (d_6-DMSO): δ 1.87 (s, 3H, C**H**$_3$), 2.92 (m, 1H, C**H**H), 3.12 (m, 1H, CH**H**), 4.49 (q, 1H, C**H**), 7.33 (m, 5H, Ar**H**), 8.33 (d, 1H, N**H**)

^{13}C NMR (d_6-DMSO): δ 22.68 (**C**H$_3$), 37.17 (**C**H$_2$), 54.06 (**C**H), 126.94 (Ar), 128.69 (Ar), 129.46 (Ar), 138.03 (Ar), 170.23 (**C**O), 173.65 (**C**O)

5.6.9 Testing recovery of organics from the C_{18} cartridge during sample workup (from section 4.2.3)

A solution of Z-L-alanine (25mM) in 1mL of 0.25M PIPES (pH 6.8) was run through a C_{18} cartridge, which was then washed with 1.5mL water. The water was collected and run through the HPLC; only a very small amount of Z-alanine was detected. The column was washed with 3x1mL of acetonitrile, with each of the three fractions being separately collected and analysed by HPLC. The first fraction showed a large Z-alanine peak when run through the HPLC, the second fraction showed a much smaller peak and the third fraction showed virtually no peak. This experiment was repeated with a saturated solution of Z-DL-alanyl-glycine methyl ester in 0.25M PIPES, and gave the same result. 2mL of acetonitrile were therefore used to elute the organics from the cartridge during sample workup.

5.6.10 Investigating oxidative catalysis during workup (from section 4.2.3)

All manipulations involving sample preparation and incubation were carried out anaerobically. Two solutions were generated: a 1M PIPES (pH 6.80) solution containing both Z-L-alanine thioacid (80mM) and the L-alanine methyl ester (80mM), and a solution of ferrous sulfate (80mM) in water. Aliquots of each solution (0.25mL) were transferred to the reaction vessels so that the resulting 0.5mL reaction solutions contained ferrous ions, the amino acid methyl ester nucleophile and the Z-

amino thioacid at concentrations of 40mM each, with a PIPES concentration of 500mM. 0.25mL of degassed water was added in place of the ferrous ion solution when generating blanks. Potassium cyanide solution (100μL, 2.4M) was immediately added to each reaction mixture, including those without ferrous ions (this represented a twelve-fold excess of cyanide ions over ferrous ions). Solutions were shaken, and then centrifuged at 15000rpm for 5min. The supernatants were passed through a C_{18} column and the organics were then eluted with acetonitrile (see section 5.1.2.5 for details). These acetonitrile solutions were injected into an HPLC and run at a linear gradient of 0-90% acetonitrile over 27min. Z-L-alanine eluted after 15.5min and Z-L-alanine thioacid eluted after 24min.

5.6.11 Construction of a phenanthroline-ferrous complex calibration curve[11]

A solution of ferrous sulfate (0.1mg/mL) in 1% (v/v) aqueous hydrochloric acid was used as a source of iron ions. Appropriately sized aliquots were taken and made up to a volume of 2mL with 1% (v/v) aqueous hydrochloric acid. 2mL aliquots of a 10% (w/v) hydroxyl ammonium chloride solution were added to reduce any ferric ions to ferrous ions. 100μL aliquots of a 2M sodium acetate solution were added, and the resulting solutions transferred to 50mL volumetric flasks. 4mL aliquots of an acidic 0.25% phenanthroline solution (0.5g of phenanthroline in 200mL 0.1M hydrochloric acid) were added, and the volumes made up to 50mL with water. The pH of the resulting solutions were about 3. Absorption values at 512nm were measured after 1min of mixing. The calibration curve constructed from these results is shown below (figure 5.1).

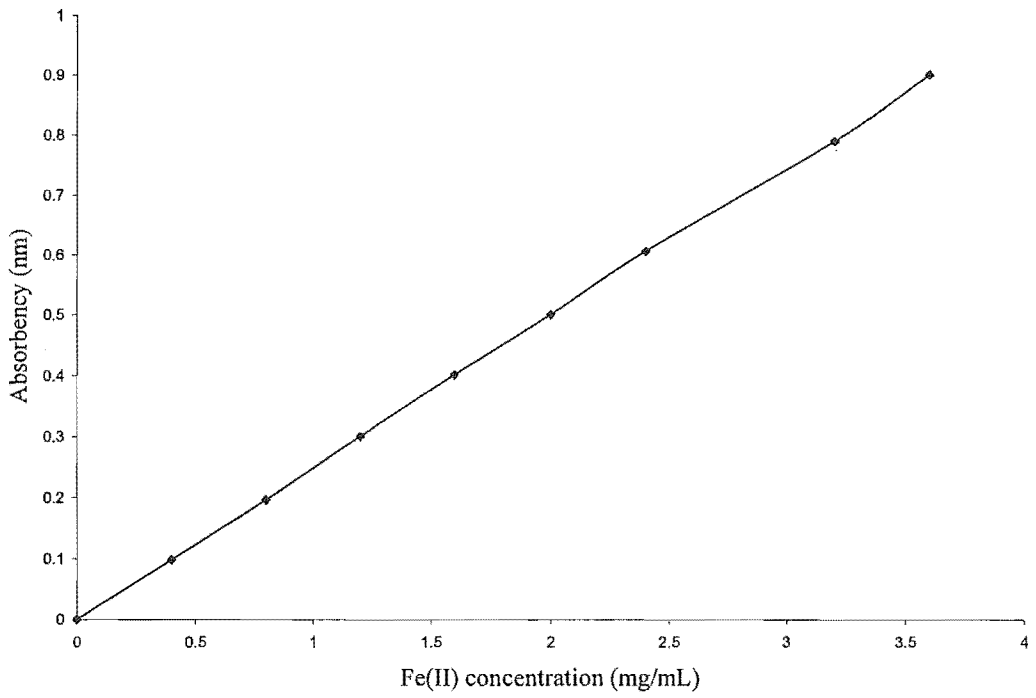

Figure 5.1: Calibration curve for a phenanthroline-based assay for Fe(II) concentration

5.6.12 Assaying for Fe(II) concentration before and after incubation (from section 4.2.4)

All manipulations involving sample preparation and incubation were carried out anaerobically. Ferrous sulfate (approximately 5mg) was weighed into sets of 6 reaction vials, and the weight accurately recorded. Two blanks, which didn't include any ferrous ions, were included in each run. The vials were degassed and 0.5mL of a degassed 1M PIPES solution was added to each. Four vials, one of which was a blank, were immediately assayed for ferrous ion concentrations: the suba seals were removed and the entire vial and all of it's contents dropped into a beaker containing 0.25% phenanthroline solution (10mL) and deionised water (10mL). The characteristic red colour developed immediately. This solution was transferred to a 250mL volumetric flask, and the beaker and reaction vial were washed several times with water which was also transferred to the volumetric flask. The solution was made up to 250mL with deionised water, mixed thoroughly, and absorption readings at 512nm taken. The blank was used to calibrate the colourimeter.

The remaining tubes that hadn't been assayed immediately were incubated at 45°C for 24hr, before being assayed for ferrous ion concentrations in a manner identical to that described above.

5.6.13 Experimental from section 4.2.5

Hampton-type experiments:

All manipulations involving sample preparation and incubation were carried out anaerobically. Two solutions were generated: a 1M PIPES (pH 6.80) solution containing both Z-L-alanine thioacid (80mM) and the L-alanine methyl ester (80mM), and a solution of ferrous sulfate (80mM) in water. 0.25mL of each solution was transferred to reaction vessels so that the resulting 0.5mL reaction solutions contained ferrous ions, the amino acid methyl ester nucleophile and the Z-amino thioacid at concentrations of 40mM each, with a PIPES concentration of 500mM. Degassed (0.25mL) water was added in place of the ferrous ion solution when generating blanks. Reaction vials were incubated in argon-filled chambers at 45°C for 24hrs or 65°C for 3days. At the end of incubation, 100μL of a 2.4M potassium cyanide solution was added to each reaction mixture, including those without ferrous ions. This represented a twelve-fold excess of cyanide ions over ferrous ions. Solutions were shaken, and then centrifuged at 15000rpm for 5min. The supernatants were passed through a C_{18} column and the organics were then eluted with acetonitrile (see section 5.1.3 for details). These acetonitrile solutions were filtered, then injected onto an HPLC and run at a linear gradient of 0-90% acetonitrile over 27min. Using this protocol and solutions of reference compounds, it was shown that Z-L-alanine eluted after 15.5min, Z-L-alanyl-L-alanine methyl ester eluted after 16.5min, and Z-L-alanine thioacid eluted after 24min.

This reaction was repeated without degassing the reaction solutions, to obtain results that are reported in section 4.4.

Testing dipeptide stability under harsher conditions:

A saturated solution of the relatively insoluble Z-alanyl-alanine methyl ester dipeptide in 200mM PIPES (pH 6.8) was generated. This solution was degassed and added to a degassed reaction vial containing ferrous sulfate (5.55mg) so that the final

concentration of ferrous ions in solution was 40mM. This solution was incubated at 65°C for 3 days, then worked up and analysed as per the above protocol.

5.6.14 Experimental from section 4.2.6

All manipulations involving sample preparation and incubation were carried out anaerobically. A solution of thioacetic acid (80mM) in 1M PIPES (pH 6.8) was generated. Because of its volatility, pure thioacetic acid could not be degassed. It was therefore first dissolved in a degassed buffer solution, before placing it under vacuum. An appropriately sized aliquot of the resulting solution was transferred to a degassed L-phenylalanine-containing flask, to give a solution containing of phenylalanine (80mM) and thioacetic acid (80mM). A solution of ferrous sulfate in water (80mM) was also generated. Aliquots (0.25mL) of each solution were transferred to degassed reaction vessels so that the resulting 0.5mL reaction solutions contained ferrous ions, L-phenylalanine and thioacetic acid at concentrations of 40mM each, with a PIPES concentration of 500mM. Degassed water (0.25mL) was added to blanks, as a substitute for the ferrous ion solution. Reaction vials were incubated in argon-filled chambers at 50°C for 24hrs. At the end of incubation, 100μL of a 2.4M potassium cyanide solution was added to each reaction mixture, including those without ferrous ions. This represented a twelve-fold excess of cyanide ions over ferrous ions. Solutions were shaken, and then centrifuged at 15000rpm for 5min. The supernatants were passed through a C_{18} column, and the organics were then eluted with acetonitrile (see section 5.1.3 for details). These acetonitrile solutions were injected into an HPLC and run at a linear gradient of 0-36% acetonitrile over 10min. Using this protocol and solutions of reference compounds, it was shown that L-phenylalanine eluted after 7.5min and *N*-acetyl eluted after 12min.

5.6.15 Experimental from section 4.2.7

Liu and Orgel experiment:

A solution of thioacetic acid (150mM) and HEPES buffer (400mM) was used to dissolve two samples of phenylalanine to give two solutions, one containing 150mM of L-phenylalanine and the other 50mM. 0.75mL aliquots of these solutions were added to 0.75mL aliquots of an aqueous solution of potassium ferricyanide(300mM). The final reaction solutions therefore contained ferricyanide ions (150mM), thioacetic acid (75mM) and L-phenylalanine (either 25mM or 75mM), and were buffered to a

pH of 6.35 with HEPES (200mM). These solutions were stirred at room temperature, and 0.5mL mL aliquots were taken after 20min and 15hrs. The aliquots were passed through a C_{18} column, and the organics eluted with acetonitrile (see section 5.2.3 for details). The acetonitrile solutions were analysed by HPLC with a linear gradient of 0-36% acetonitrile over 10min. Samples to be analysed by HPLC/MS were also analysed as acetonitrile solutions.

Oxidative acylation with Z-amino thioacids and amino acid methyl esters:
Two aqueous solutions were generated; one contained L-alanine methyl ester (80mM), Z-L-alanine thioacid (80mM) and PIPES buffer (1M, pH 6.8), and the other was an aqueous solution of potassium ferricyanide (160mM). 0.5mL of each solution was transferred to a reaction vessel, so that the resulting reaction solution contained 40mM L-alanine methyl ester, 40mM Z-L-alanine thioacid, 80mM potassium ferricyanide and 500mM PIPES (pH 6.8). This solution was stirred at room temperature and a 0.5mL aliquot taken after 20hrs. This aliquot was passed through a C_{18} column, and the organics eluted with acetonitrile (see section 5.2.3 for details). This acetonitrile solution was injected into an HPLC and run at a linear gradient of 0-90% acetonitrile over 27min. Three peaks appeared at 7.5min, 12min and 16min; the first two of which were shown (by spiking with authentic samples) to be phenylalanine and *N*-acetyl phenylalanine.

Acylation with ferric ions:
An excess of ferric chloride was added to solutions containing Z-alanine thioacid (40mM), alanine methyl ester (40mM) and 500mM PIPES (pH 6.8). Reaction mixtures were incubated for three days at 65°C. At the end of incubation, 100μL of a 2.4M potassium cyanide solution was added to the reaction mixture, which was then centrifuged at 15000rpm for 5min. The supernatant was passed through a C_{18} column and the organics were then eluted with acetonitrile (see section 5.1.3 for details). This acetonitrile solution was filtered, then injected onto an HPLC and run at a linear gradient of 0-90% acetonitrile over 27min.

5.6.16 Experimental from section 4.3

All manipulations involving sample preparation and incubation were carried out anaerobically. 2 solutions were generated: a 1M PIPES (pH 6.80) solution containing

both Z-L-alanine thioacid (80mM each) and L-alanine methyl ester (80mM each), and a solution of nickel chloride in water (80mM). 0.25mL of each solution was transferred to degassed reaction vessels so that the resulting 0.5mL reaction solutions contained the metal ion, amine nucleophile and thioacid at concentrations of 40mM each, with a buffer concentration of 500mM. 0.25mL of degassed water was added to the "blank", as a substitute for the metal ion solutions. Alternatively, solutions were not degassed during sample preparation. Reaction vials were incubated in argon-filled chambers at 65°C for 3days. At the end of incubation, 100μL of a 2.4M potassium cyanide solution was added to each reaction mixture, including those without ferrous ions. This represented a twelve-fold excess of cyanide ions over ferrous ions. Solutions were shaken, and then centrifuged at 15000rpm for 5min. The supernatants were passed through a C_{18} column and the organics were then eluted with acetonitrile (see section 5.1.3 for details). These acetonitrile solutions were filtered, then injected onto an HPLC and run at a linear gradient of 0-90% acetonitrile over 27min.

5.6.17 Experimental from section 4.4

Two solutions were generated: one containing Z-alanine thioacid (80mM), alanine methyl ester (80mM) and sodium hydrogen sulfide (90mM) in 1M PIPES (pH 6.8); and the second containing ferrous sulfate (80mM), nickel chloride (80mM) or ferrous sulfate (40mM) and nickel sulfide (40mM) in water. 0.25mL of each of the two types of solution was transferred to degassed reaction vessels. Metal sulfide precipitates fell out of solution immediately; the iron sulfide precipitates were dark black, and the nickel sulfide precipitates were a dark silver colour. The resulting 0.5mL reaction mixtures contained the thioacid and amine nucleophile at concentrations of 40mM (the same as in experiments involving the free metal ions in solution) and FeS, NiS or (Fe,Ni)S precipitates with equimolar amounts of the metal ion. Reaction mixtures were incubated at 65°C for 3 days. At the end of incubation, 100μL of a 2.4M potassium cyanide solution was added to each reaction mixture, including those without ferrous ions. This represented a twelve-fold excess of cyanide ions over metal ions. Solutions were shaken, and then centrifuged at 15000rpm for 5min. The supernatants were passed through a C_{18} column and the organics were then eluted with acetonitrile (see section 5.1.3 for details). These acetonitrile solutions were

filtered, then injected onto an HPLC and run at a linear gradient of 0-90% acetonitrile over 27min.

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